Single Embryo Transfer:
Clinical and Immunological aspects

Single Embryo Transfer: Clinical and Immunological aspects

Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus Prof. dr. C.W.P.M. Blom,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op woensdag 1 juni 2005
des namiddags om 3.30 uur precies
door

Henrike Getrud Maria Lukassen

geboren op 13 november 1970
te Arnhem

Promotores:	Prof. dr. D.D.M. Braat
Co-promotores:	Dr. I. Joosten Dr. J.A.M. Kremer
Manuscriptcommissie:	Prof. dr. B.E. de Pauw Prof. dr. L.A.A. Kollée Prof. dr. J.M.R. Gerris (Middelheim Ziekenhuis, Antwerpen)

ISBN 00 0000 000 0

Omslag en boekverzorging: Peter Tychon, Wychen
Printed by drukkerij MacDonald / SSN b.v., Nijmegen

Contents

1 *General Introduction*

Part I: Clinical Aspects

In Vitro Fertilization

Ten percent of Dutch couples suffer from a fertility disorder (1;2). Of these couples 50% are treated with IVF or Intracytoplasmic Sperm Injection (ICSI) treatment. Nowadays, in the Netherlands one out of 55 children is conceived through IVF or ICSI (3).

Infertility has been well known since ancient times (4). As long as physicians were unable to help women satisfy their basic instincts for procreation and motherhood, infertility was considered a devine infliction treated by equal measures of witchcraft, myth and good faith. Not until the second half of the 20th century did effective treatment of infertility replace magic and belief. During the fifth decade of the 20th century, clomiphene citrate emerged as the first effective promoter of fertility. Within the next 20 years, gonadotrophins were produced and used to treat infertile couples. These hormones induced multiple follicle growth of the ovaries. By the early 1980s it became world wide possible to retrieve oocytes, fertilize them with spermatozoa outside the body and transfer one or more embryos in the uterus, the so-called in vitro fertilization (IVF) treatment.

In 1978 the first successful pregnancy resulting from IVF occurred during an unstimulated normal menstrual cycle (5). IVF in the natural cycle was soon replaced by IVF with ovarian hyperstimulation. The use of exogenous gonadotrophins resulted in higher numbers of embryos available for transfer, while gonadotrophin-releasing hormone analogues decreased the cancellation rate (6;7). Both measures led to better pregnancy rates.

At present the following IVF protocol is used at the IVF department of the University Medical Center Nijmegen. Pituitary down-regulation (long protocol) is achieved using a GnRH analogue (Decapeptyl®; Ferring, the Netherlands). Multiple follicular stimulation is realized by recombinant follicle-stimulating hormone (Puregon®; Organon, Oss, the Netherlands). Thirty-six hours after hCG injection (Pregnyl®; Organon, Oss, the Netherlands) transvaginal oocyte retrieval is performed under

systemic analgesia (10 mg oxazepam orally and 1mg alfentanyl, i.v.). The retrieved oocytes are inseminated or ICSI is performed according to the method described by Van Steirteghem and colleagues (8). The morning following injection or insemination fertilization of the oocytes is judged. Three days after oocyte retrieval one or two best quality embryos are transferred. The luteal phase is supported by three doses of progesterone 200 mg (Progestan®; Organon, Oss, the Netherlands) intravaginally daily for fifteen days starting from the day of oocyte retrieval.

Iatrogenic Multiple Pregnancies

By the early 1980s the number of twins increased dramatically in the Netherlands, from 10/1000 births to 18/1000 births in the year 2000, and this number is still increasing. Also the number of triplets and higher order births increased from 1980 (0.15/1000 births) to the early 1990s (0.6/1000 births), but this number is decreasing since 1992 (www.cbs.nl). Increase in maternal age and medical management of subfertility are the most important factors that contribute to the rapid increase in multiple birth rates (9;10). Older women are at higher risk of spontaneous multiple birth than younger women due to higher basal FSH levels (11;12) and over the past 25 years a trend towards delayed childbearing has been observed in Europe. The Netherlands even took the lead. The mean age at which the Dutch women have their first child increased from 24.3 years in 1970 to 29.2 years in 2001 (www.cbs.nl).

As mentioned before, the medical management of subfertility is also an important contributor to the huge increase in multiple births. In ovulation induction as well as in mild hyperstimulation and IUI (intra uterine insemination) multiple follicular growth is positively correlated with a higher multiple pregnancy rate. The number of embryos obtained after IVF is a good prognostic value for the chance of pregnancy, whereas the multiple pregnancy rate is a direct consequence of the number of embryos transferred (13). Due to controlled ovarian (hyper)stimulation, with or without intrauterine inseminations, the risk of multiple births varies from 8% with clomiphene to 20-30% with gonadotrophins (14). In case of IVF or related assisted reproductive technologies (ART) the percentage of twins is 24% and triplets 2% in Europe (15). In the Netherlands the number of IVF twins was 21.8% and triplets 0.8% in 2003

(www.nvog.nl). In our country probably half the twin maternities are a result of ovarian stimulation or IVF and the other half is accounted for the increase in maternal age (16).

Templeton showed that the chances of live births after IVF are related to the number of oocytes fertilized, presumably because of the greater number of embryos to select for transfer or as expression of better ovarian response. When more than four oocytes are fertilized and available for transfer, the woman's chance of a birth is not diminished by transferring only two embryos. Transferring three or more embryos increases the risk of multiple births (13). Since the publication of this article, in the Netherlands a maximum of two embryos are routinely transferred per cycle to prevent higher-order multiple pregnancies. The risk for a twin pregnancy with this regime, however, is still 20-35% (17), which is a 15-fold increase relative to the risk of 1.6% after natural conception (17;18).

In the Netherlands in 2003 IVF resulted in 20 triplets, 806 twins and 2888 singletons. The contribution of IVF to the total number of triplets was 28.6% (20/70). Out of a total number of 3616 twins the number of IVF-twins was 806 (22.3%) (www.nvog.nl).

Risks of Iatrogenic Multiple Pregnancies

The high rate of multiple births resulting from in vitro fertilization is a major health isssue. Twin pregnancies cause more maternal and perinatal mortality and morbidity (19-21), and imposes a steep burden on government expenses and health services (21-23). Furthermore, it should not be forgotten the long-term health consequences and the psychosocial problems related to multiple births.

Maternal complications

One of the major maternal complications associated with multiple pregnancies is pregnancy-induced hypertension. Even after adjustment for age and parity, most studies indicate that the risk of developing severe hypertension is two to three times greater for a twin pregnancy than for a singleton pregnancy. Pre-eclampsia is also three times more common in twin pregnancies than in singletons, among primigravid women carrying a twin pregnancy this risk is even five times higher (24). The true incidence of pre-eclampsia among patients with multiple pregnancies may

be underestimated because of the increased number of preterm deliveries before the onset of clinical symptoms of pre-eclampsia (25). Douglas and colleagues found in the United Kingdom a relative risk of 6.0 (95% CI 4.1-8.9) for eclampsia in multiple pregnancies as compared with singleton pregnancies (26).

Anemia, antepartum bleeding from placenta previa and abruptio placentae are more common in multiple pregnancies. Greater amounts of intrapartum blood loss as a result of higher incidence of surgical intervention and postpartum hemorrhage due to atony occurs more often in women with multiple pregnancies (27).

Multiple gestations are associated with an increased rate of deliveries by Caesarean section. After Caesarean section an increased rate of pulmonary emboli, hemorrhage, postpartum infection, wound infection and prolonged hospitalization is observed. The maternal death rate associated with Caesarean sections is two to four times higher than with vaginal delivery (24).

Preterm labour and preterm premature rupture of membranes occur more often in multiple pregnancies than in singletons. The margins of safety of tocolytic therapy are narrower for mothers of multiples than for mothers with singleton pregnancies because of increased maternal blood volume and cardiac output. The side effects of beta-sympathomimetics occur more frequently and are more severe. Careful monitoring of cardiovascular parameters en strictly fluid management are critical in preventing pulmonary oedema (28).

The increased risk for serious maternal morbidity leads to a higher incidence of maternal mortality. Senat and colleagues estimated that, in France, the maternal mortality was more than two times higher in multiple versus singleton pregnancies (10.2 versus 4.4 per 100,000 live births). For Europe these figures were 14.9 versus 5.2 (odds ratio 2.9, 95% CI 1.4-6.1) (24).

Perinatal and long-term complications

Infants from multiple pregnancies are more likely to be born prematurely and with a lower birth weight. Martin et al. showed that 57.4% of the twins was born prematurely (<37 weeks) and that the mean gestational age was 32.0 (SD 4.0) weeks. Singletons, however, were born at a mean gestational age of 38.8 (SD 2.5) weeks, 10.4% was born prematurely. The mean birth weight of twins was 2353 (SD 647) gram, and 54.9% had a low

birth weight (<2500 gram). Singletons had a mean birth weight of 3339 (SD 573) gram, and 6.0% had a low birth weight (29). Two other reports showed the same results (21;30). Obviously many perinatal complications can be addressed to prematurity and low birth weight. Treatment in neonatal intensive care units (NICU) was required for 15% of singletons and 48% of twins (22). Twins have a fivefold increase in the risk of fetal death and even a sevenfold increase in the risk of neonatal death when compared to singletons (31).

There is an increased risk of long-term medical and developmental problems, in particular neurological impairment, in children from multiple pregnancies. The risk of cerebral palsy in twins is approximately five times higher than that in singletons, on the basis of 1000 first-year survivors, but the risk increases to 8.3 times when calculated per 1000 pregnancies (32). The risk of handicaps increases also with the number of fetuses. Twins have a 1.4-fold increased risk for all handicaps compared with singletons (33).

Economic implications

Multiple pregnancies have been shown to require more medical care than singleton pregnancies and therefore generate more costs. Multiple pregnancies are associated with higher maternal costs (hospital admission, frequently monitoring, preventing and treating complications), higher delivery charges (increased Caesarean section rate, longer hospital admission), higher neonatal costs (medical problems related to prematurity and low birth weight, higher NICU admission rate), and higher childhood costs (more physical and mental handicaps) (18;21;22).

Ettner and colleagues indicated that gestational age accounts for 50% of the increase in total charges due to multiple gestation, birth weight accounts for 40%, and neither gestational age nor birth weight could account for the remaining 10% (34). Callahan et al. estimated total family charge as 9,845 dollar for a singleton delivery and 37,947 dollar for twins (18,974 dollar per baby). This study was performed at Brigham and Women's Hospital in Boston from 1986 to 1991 (22). Wolner-Hannsen et al. chose another approach. They compared the costs of an IVF treatment with single embryo transfer (SET) and double embryo transfer (DET), with a live birth rate of 21% in the SET and 25% in the DET group and a twin pregnancy rate in the DET group of 24%. The total costs for the DET strategy were 4 times higher than for the SET strategy (23).

Obviously, the costs of IVF treatment and hospital admission as well as the IVF results and prevalence of complications differ from country to country and even within one country.

We must be aware of the fact that costs may be higher for IVF twins than for non-IVF twins (35), because IVF-pregnancies (singletons and multiples) are at greater risk for obstetric and perinatal complications than spontaneously conceived pregnancies (36-41).

Psychosocial impact

The psychological and social impact of multiples on the host family must not be underestimated. Many parents are unprepared for the impact that the birth of a twin has on the relationship with their partner. Couples of IVF twins indicated that they found parenting considerably less rewarding than they had expected (43). Furthermore, mothers of IVF twins experience more parenting stress as compared with mothers of IVF singletons and naturally conceived singletons (43-45). Parents of multiples suffer more often from fatigue and depression and are more likely to get divorced (46;47).

Mothers of premature twins are less responsive to their babies than are mothers of singletons; multiples may therefore be particularly vulnerable to the psychological consequences of obstetric complications (48). Even when the children are healthy, factors as shared parental attention, triadic communication, and the unique relationship between twins themselves may adversely affect social skills and language development (49).

However, we also have to consider that patients do not always share their physicians' concerns about multiple pregnancies or are more willing to accept the associated risks (50). The ideal outcome of IVF from a clinical perspective would be a healthy singleton child, whereas many infertile couples are hoping for a ready-made family and 'two for the price of one'. Ryan and colleagues described in a prospective analysis that 20% of the IVF patients listed a multiple birth as their most desired outcome of IVF treatment. Only a minority of patients (30%) was aware of all of the risks of a twin pregnancy and this lack of knowledge was predictive of the desire for multiple births. Nulliparity, lower family income, younger patients age, prior evaluation for infertility, and longer duration of infertility were also associated with the desire for multiple births. Only nulliparity and lower family income were independently associated

with this desire (51). Because patients are actively involved in clinical decision-making, for example implementation of SET, understanding patient desires and knowledge about multiple births is important. It seems that risk education may play an important role in counselling IVF patients to understand and accept the need for SET.

Avoiding Multiple Pregnancies in IVF

Single Embryo Transfer

As will be clear from the above paragraphs, multiple pregnancies are the most serious complication after IVF treatment. Nowadays, most clinics in Europe transfer two embryos per cycle, to reduce chances for high-order multiple pregnancies (13). Also in the Netherlands routinely a maximum of two embryos per cycle are transferred. In Europe, the multiple birth rate in 1997 was 30%, with twins at 26% and triplets at 4% (52). The only solution to diminish the number of multiple births dramatically is single embryo transfer (SET). There is a conflict of interest between the ambitions of single birth after IVF by SET on the one hand and the emotional and economical aspects of the possibly lower pregnancy rate on the other.

One solution to overcome this dilemma is to select embryos with a higher potential for implantation. At the present time, cytoplasmic fragmentation and cleavage rate are the determinants of embryo selection for transfer. Although high quality day-3 embryos possess a greater potential for establishing a pregnancy, these selection criteria are rather poor predictors of subsequent implantation and pregnancy rates. When day-3 embryos are cultured for a prolonged period of time until the blastocyst stage, only viable blastocysts with a high developmental potential will survive. The implantation rate of these day-5 blastocysts appears to be higher, up to 50% (53-55). With optimal culture conditions about 50% of the embryos will develop into blastocysts (53;56). A randomized controlled trial comparing day 5 versus day 3 transfer of two embryos did not result in a significant difference in pregnancy rate between both groups (57). Even when blastocysts are morphologically graded as optimal, 50% appears to be aneuploid (58). With the introduction of preimplantation genetic screening (PGS) for aneuploidy, it has become possible to analyze the ploidy status of several chromosomes from in-vitro cultured

embryos (59). The exclusion of aneuploid embryos before transfer improves the implantation rate (60) and reduces the abortion rate (61). A disadvantage of this method is the manipulation of the embryo risking damage and loss of embryos. It is important to study the cost-effectiveness of PGS in IVF before introducing this as a standard procedure. Furthermore, it may raise ethical objection like eugenics.

In order to reduce the multiple pregnancy rate substantially, while maintaining an acceptable overall pregnancy rate, the patients at risk for multiple pregnancy and their treatment characteristics have to be identified. Retrospective studies indicated age, number of embryos available and quality of embryos as the most important predictors for multiple births (62-64).

Up to now only four randomized controlled trials comparing single embryo transfer (SET) and double embryo transfer (DET) have been published (65-68). The study of Gerris et al. was performed in 53 patients younger than 34 years of age. At least two top quality embryos had to be available. The difference in the ongoing pregnancy rates between the SET group (38.5%) and the DET group (74.1%) was not significant (65). The study of Martikainen et al. was performed in 144 patients with at least four good quality embryos. There was also no statistical difference in cumulative live birth rate (fresh and frozen cycles) between the SET group (39%) and the DET group (51%) (66). Gardner et al. randomized 48 IVF patients with at least 10 follicles > 12 mm on the day of hCG administration to either the transfer of one blastocyst or two blastocysts on day 5. There was no significant difference in ongoing pregnancy rate between single blastocyst transfer (61%) and double blastocyst transfer (76%) (67). Finally, Thurin et al. performed a multi-centre randomized trial in 661 women who were less than 36 years of age and had at least two good quality embryos available. The cumulative live birth rate in the SET group (one fresh SET and one frozen SET cycle) was 38.8% compared with 42.9% in the DET group (without a frozen DET cycle) ($p = 0.3$) (68). The multiple birth rate was 33.1% in the DET and 0.8% in the SET group. The live birth rate after only one fresh-embryo cycle was significantly lower in the SET group than in the DET group, 27.6% and 42.9% respectively.

In Finland elective single embryo transfer is already successfully implemented in daily practice, where DET is only considered in patients with two unsuccessful IVF cycles or poor embryo quality. In a retrospective

Finnish study 1871 IVF cycles carried out from 1997 to 2001 were analyzed. The number of SET cycles increased from 11% to 56%. The clinical pregnancy rate per transfer increased from 28.3% to 30.3%, while the multiple birth rate dropped from 25% to 7.5% (69). In Belgium, Gerris and colleagues studied also the effect of elective SET in a large group of patients (n=1559). They showed in a retrospective cohort analysis over a four year period that at least one top embryo (4 or 5 blastomeres on day two, at least 7 blastomeres on day three, <20% fragments and the absence of multinucleated blastomeres) could be transferred in ~70% of all cycles. Over these four years SET increased from 13% to 31%, whereas the ongoing pregnancy rate per retrieval did not change (35.9% to 31.0%), but the multiple pregnancy rate decreased with almost 50% (33.6% to 18.6%) (70).

Natural Cycle IVF

IVF treatment is an effective method for many couples with various causes of infertility to become pregnant. The first successful pregnancy resulting from IVF occurred during a natural menstrual cycle (5). Since then, ovarian stimulation has been widely used in assisted reproduction programs all over the world. The advantages of ovarian hyperstimulation are increased numbers of oocytes and subsequent embryos available for transfer, which is associated with a higher chance of pregnancy (6;71). Better cycle control was achieved by pituitary desensitization with the use of gonadotrophin-releasing hormone analogues, which improved the pregnancy rate after IVF even more (7).

There are major concerns, however, about the risks of ovarian stimulation. In ovulation induction, intra uterine insemination (if combined with ovarian hyperstimulation) and IVF the most important risk is the high incidence of multiple pregnancies, with its attendant obstetric and neonatal problems (see above). Another serious risk, predominantly occurring in IVF, is the ovarian hyperstimulation syndrome (OHSS), which is a life threatening complication (72;73). Although available data are reassuring, concern remains about the possible increased risk of developing ovarian, endometrial or breast cancer after repeated ovarian stimulations (74-79). Legal and ethical dilemmas concerning the storage and disposal of spare embryos have to be taken into account. A decrease in endometrial receptivity as a result of hormone treatment is suggested, but this remains controversial (80-83). Finally, natural cycle IVF offer a low-cost alternative that may be more accessible to patients (84;85).

These disadvantages have led to the revival of IVF in the natural cycle by several research groups, especially since the IVF technique and LH monitoring have been improved over the years. Pelinck et al. performed a systematic review to determine the effectiveness of natural cycle IVF. The 20 selected studies described a total of 1800 natural cycles of IVF. The cancellation rate of oocyte retrieval was 28.9% and embryo transfer was performed in 45.5% of the cycles. The ongoing pregnancy rate was 7.2% per cycle and 15.8% per embryo transfer (86). Daya and colleagues calculated that the incremental direct medical treatment cost incurred per live birth after IVF when using stimulated cycles, compared to natural cycles, is approximately 48,000 dollar. Moreover, this protocol can be performed on a monthly basis, in contrast to IVF after stimulated cycles that takes in general three months, because the ovaries need to recover from hormonal stimulation. For up to three unstimulated cycles no decrease in pregnancy rate per cycle is observed (87). So, it is suggested that in the same time span natural cycle IVF (after three consecutive cycles) might be even effective as one stimulated cycle IVF. A cost-effectiveness study comparing (multiple) natural cycle IVF treatment with SET after stimulated cycle IVF is warranted.

It has been suggested that IVF in the natural cycle can be best performed in patients with tubal factor infertility, as in these couples both male and female gametes are supposed to be normal (88). However, in case of severe oligozoospermia, the efficacy of ICSI in the natural cycle has never been investigated serially and in a systematic way before.

Selective Embryo Reduction

Selective embryo reduction is proposed by some individuals as a manner of dealing with high-order multiple gestations. The most commonly used technique involves the insertion of a needle into the fetal thorax and the injection of KCL (potassium-chloride) into the fetal heart to achieve cardiac arrest. Individual clinic-derived data are conflicting with regard to the improvement in perinatal outcome achieved by embryo reduction of triplets to twins. However, with four or more fetuses, the improvement in outcome of selective embryo reduction is dramatic (89). Selective embryo reduction is not without risks, however. Evans and colleagues reported that only 84% of pregnancies resulted in an ongoing pregnancy after embryo reduction, and 5% of these pregnancies resulted in an extreme preterm labour (25-28 weeks of gestation) (90).

The preplanned use of selective embryo reduction to 'correct' overly aggressive fertility treatment should be rigorously discouraged as an unethical and inappropriate use of this procedure. Retrospective studies show that many couples (32%) are ultimately uncomfortable with their decision to reduce (91). The sequence of producing iatrogenic high-order multiples that are subsequently submitted to iatrogenic termination is considered as the ultimate paradox of infertility treatments.

When SET might be put into practice in order to decrease the number of IVF-twins and higher order multiple births, the pregnancy rate could drop slightly as well. To facilitate the implementation of SET, it is important to improve the pregnancy rate. One way to accomplish this is a cryopreservation programm with good clinical results. Another way to increase the live birth rate is to perform more fundamental research on the process of implantation in order to improve this critical stage in pregnancy. The fetus is considered to be a semi-allograft that has to be accepted by the maternal immune system. Therefore, we believe that it is important to gain more insight into the immunological interaction between the fetus and the mother during implantation. In this thesis we focused on natural killer (NK) cells, because they play an important role in human implantation.

Part II: Immunological Aspects

Immunology and Reproduction

Ovary

Unlike the testes, the ovary does not constitute an immunologically privileged site. Resident ovarian monocytes-macrophages, lymphocytes and granulocytes can be observed at various stages of the ovarian life cycle and actively participate in the functional and structural changes of the follicle and corpus luteum development. The vascular network around the dominant follicle, located in the theca-layer, shows an increased blood flow and increased permeability to attract leukocytes into the follicle tissue through cytokines and chemokines leading to successful ovulation (92). Until now in follicular fluid the presence and function of many cytokines have been determined (93-99). Follicular fluid provides the

environment in which oocyte maturation occurs and may therefore influence the quality of the oocyte, and thus be linked to fertilization and early embryonic development. However, there is only limited information from dated studies evaluating the immune cells (i.e. leukocytes) present in FF responsible for the production of those cytokines (99-105).

In case of IVF multiple follicle growth is induced by hormonal stimulation. To enhance the pregnancy rate the best quality embryo(s) are selected for transfer into the uterus. At the present time, morphology and cleavage rates are the determinants of embryo selection for transfer. Unfortunately, both are poor predictors of subsequent implantation and pregnancy. Therefore, to improve the implantation rate we are eager to find better prognostic markers for embryo quality in either follicular fluid or in embryo culture supernatant (ECS). The leukocytes present in follicular fluid might influence growth and development of oocytes and subsequent embryos and could therefore be of value as a prognostic marker in embryo selection.

Endometrium

Even more than in the ovary immunological processes play a role in the endometrium. The uterus is uniquely adapted to support a pregnancy, while retaining the barrier function that is characteristic of all mucosal tissues. The capacity to distinguish between, and appropriately respond to, the array of foreign entities to which it is exposed is achieved through a highly specialized local immune system. Specific mechanisms acting to elicit immunological tolerance in the case of semen and embryos co-exist with active immunity to defend the uterus from pathogenical bacteria and viruses.

Leukocytes play an important role in remodelling the endometrium during every menstrual cycle. This remodelling is orchestrated by the ovarian steroid hormones, estrogen and progesterone. In the proliferative phase T cells, macrophages, a few B cells and a few NK cells are present in the uterine mucosa. T cells and macrophages remain at much the same density throughout the luteal phase and during the process of decidualization. In contrast, NK cell numbers increase dramatically in the luteal phase and remain at a high level during first trimester pregnancy. In the decidua, 70% of the infiltrating leukocytes are NK cells, together with macrophages (20%) and a small number of T cells (10%) (106). The number of NK cells is particularly high in the decidua basalis at the

site of embryo implantation (42;106-109). Uterine NK cell death occurs as soon as progesterone levels drop, either pre-menstrually or when a pregnancy fails (109).

Ovarian stimulation for IVF treatment results in supraphysiological concentrations of sex steroids during the follicular and luteal phase. The effect on endometrial receptivity remains controversial. In IVF both positive and negative effects on pregnancy rates have been reported (80-83). During the menstrual cycle as well as throughout pregnancy the leukocyte numbers vary, therefore an interaction with the leukocytes with the sex steroids progesterone and oestradiol is suggested, although it is not clear yet whether this is a direct or an indirect mechanism. DeLoia et al. have shown that the total number of lymphocytes in endometrium as well as in peripheral blood, particularly the NK cells, increases under influence of supraphysiological estrogen levels (110). Because we were anxious to find out whether hormonal stimulation during IVF treatment improves or deteriorates the quality of the endometrium we started a study.

Implantation

The fetus can be considered as a semi-allograft, since fetal cells contain both maternally and paternally derived antigens. Medawar postulated this immunological concept of pregnancy for the first time in 1954 (111). However, several recent developments seriously question this statement. The fetal-maternal relationship is more akin to a host versus tumour or host versus parasite relationship than a host versus graft relationship (112). The maternal-fetal relationship is a unique process representing a step-by-step, programmed interactive symbiosis.

Trophoblast invasion into the decidua results in the formation of a specialized organ, the placenta. During implantation fetal cells come in direct contact with maternal cells, therefore the immune system of the mother has to adapt to make a successful pregnancy possible. Around 6 days after fertilization, the embryo differentiates into a blastocyst that consists of an inner cell mass that will form the embryo and an outer trophectoderm that will become the placenta and chorion. The blastocyst becomes embedded by decidualized stroma. Columns of cytotrophoblast cells (villi) emerge from the blastocyst and invade the decidua basalis. In these villi embryonic vascularisation develops. Erosion of maternal capillaries by the invading trophoblast causes the filling of

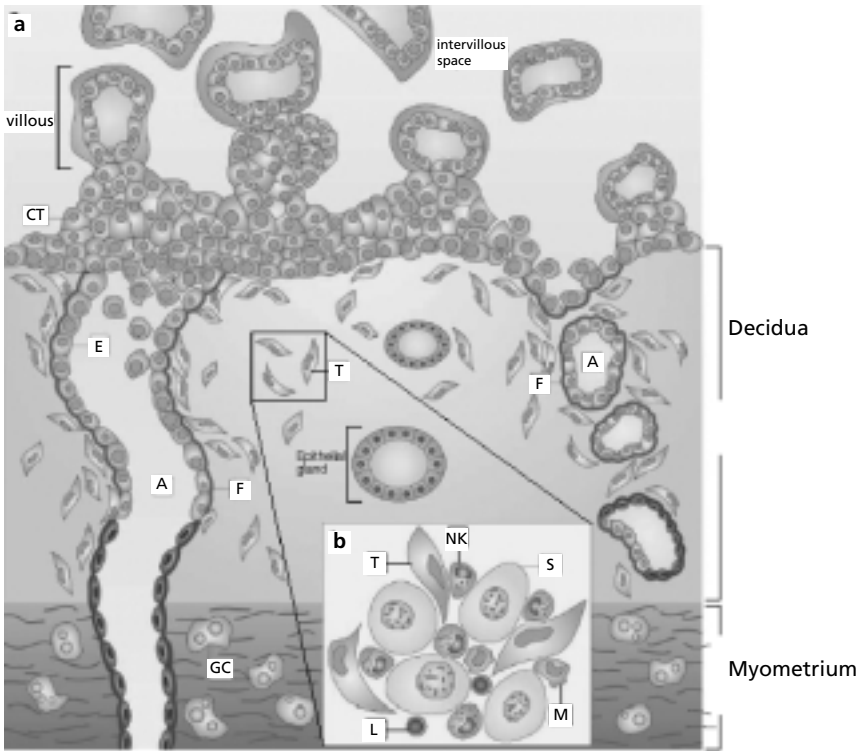


Figure 1. Anatomy of the trophoblast populations that are present at the fetal-maternal interface in the first trimester of pregnancy.
a: The placental villi are covered by villous trophoblast cells; an inner mononuclear cytotrophoblast layer covered by syncytiotrophoblast. The core of each villous contains fetal blood vessels, fibroblasts and fetal macrophages (Hofbauer cells). Maternal blood in the intervillous space reaches the placenta through the uterine spiral arteries (A). The inner layer of villous cytotrophoblast grows out at focal points to form cell columns (CT). These are prominent at the anchoring villi, where attachment to the maternal decidua occurs. At the fetal-maternal boundary, the columns form a partially continuous shell. From this shell, extravillous trophoblast cells enter the decidua as interstitial trophoblast cells (T) to encircle and destroy the arterial media, which is replaced by fibrinoid material (F). Then, endovascular trophoblast cells (E) move down the arteries in a retrograde manner to replace the endothelial cells. The trophoblast cells move as far as the inner myometrium, where they fuse to become placental-bed giant cells (GC). Extravillous trophoblast cells include cytotrophoblast cell columns and shell, interstitial and endovascular trophoblast and placental-bed giant cells. **b.** A schematic representation of the decidual stroma at the implantation site. Interstitial trophoblast cells (T) are seen between large stromal cells (S). The maternal leukocytes that are present are uterine NK cells (NK), as well as few macrophages (M) and occasional T cells (L).
From: Nature Reviews Immunology. © Dr. A. Moffett-King (109).

spaces within the syncytium with maternal blood. The fusion of these lacunae allows formation of a sponge-like network, the intervillous space. Only one layer of trophoblast cells separates the fetal and maternal blood stream making exchange of nutrients and gases possible. This is called the haemomonochorial placenta.

At the end of the first trimester of gestation a second very deep invasion (1/3 of the uterine wall) of extra-villous trophoblast takes place. Namely, there are two types of cytotrophoblast cells known as villous trophoblast and extra-villous trophoblast. These two cell populations make different contributions in establishing efficient transport between the maternal and fetal circulations. Villous trophoblast covers the chorionic villi, providing the barrier through which metabolic exchange between mother and fetus occurs. A small part of the villi are anchored to the decidua. At the tip of these anchoring villi a cell column of proliferating cytotrophoblast cells disrupts the epithelial syncytiotrophoblast layer. These cells differentiate into a distinct population of extra-villous trophoblast cells. The primary role of the extra-villous trophoblast is thought to be the regulation of maternal blood flow into the intervillous space by invasion and transformation of decidual arteries. The intramural, endovascular and interstitial trophoblast cells respectively invade the maternal arterial walls, arterial lumen and interstitium (Figure 1) (113). An important function of the invading trophoblast is to destroy the muscular walls of the uterine spiral arteries converting them into large, uncoiled vessels that are capable of high conductance and are not responding to vasoactive stimuli. These vascular adaptations provide an increased blood flow to the intervillous space, without which a variety of obstetrical problems, like miscarriage, pre-eclampsia, growth retardation and still birth can occur (114).

The maternal-fetal interface

Fetal side

Implantation of the developing embryo into the decidua of the maternal uterus is a critical event in human pregnancy. Maternal tolerance to the fetal alloantigens is essential in this stage of pregnancy. The fetus expresses the full array of its paternally inherited MHC (Major Histo-

compatibility Complex) genes (112). However, the fetus is surrounded by trophoblast cells and it is these cells that make the primary contact with maternal immune cells. These extra-embryonic cells only express a select few of the MHC antigens at their surface.

HLA molecules on trophoblast

Antigens presented on the cell surface of nucleated cells and trombocytes are called the Major Histocompatibility Complex. In humans the antigens are called 'Human Leukocyte Antigens' (HLA). The molecules can be devided into two classes. Classical class I molecules (HLA-A, HLA-B, HLA-C), non-classical class I molecules (HLA-E, HLA-F, HLA-G) which are expressed on all nucleated cells and class II molecules (HLA-DR, HLA-DQ, HLA-DP) which are only presented on antigen presenting cells, like B-cells, dendritic cells and macrophages. The non-classical class I molecules have limited polymorphism and have a restricted tissue distribution.

Remarkably, villous syncytiotrophoblast that lines the intervillous space and is in close contact with immune cells in maternal blood does not express any MHC class I antigen. These cells are presumably immunologically inert to a T cell-mediated attack. Cytolytic activation of NK cells also does not occur, although NK cells are generally activated by the absence of MHC antigens. This is probably due to lack of activating ligands on syncytiotrophoblast and protection of the surface by high levels of glycosylation.

Extra-villous trophoblast, however, express a unique limited combination of three subtypes of MHC class I antigens, HLA-C, HLA-G and HLA-E (112;115-118). HLA-F expression is also discovered on trophoblast (118), but this has not yet been confirmed by others. Extra-villous trophoblast does not express MHC class II molecules, nor does it express the two main classical MHC class I antigens, HLA-A and HLA-B. HLA-A and HLA-B are the molecules that are involved in transplant rejection.

HLA-C is a polymorphic classical MHC class I molecule. The paternal allele is expressed on the extra-villous trophoblast cell surface and may be important for allorecognition of trophoblast. HLA-C shows ubiquitous expression. A greater proportion of uterine NK cells than peripheral blood NK cells express the KIR receptor specific for HLA-C, suggestive of a role in implantation.

HLA-G is a non-classical class I molecule. HLA-G is of particularly interest, because it is only expressed on the fetal-maternal interface by invasive trophoblast cells, in amniotic fluid and on thymic epithelial cells (119). Furthermore, this molecule is characterized by a reduced polymorphism. Paternally inherited HLA-G genes expressed by the fetus would produce proteins almost identical to those of the mother. Thus, HLA-G is thought to be expressed on trophoblast without being recognized as 'foreign' by the maternal immune system. Data suggest that HLA-G expression is also induced in peripheral organs/tissues under certain pathological conditions (120;121). HLA-G can be expressed as seven different isoforms, four being membrane bound (HLA-G1 to HLA-G4) and three being soluble (HLA-G5 to HLA-G7) (122-126). These seven isoforms arise from alternative splicing of a unique primary transcript. HLA-G can act on all players of immune responses (NK cells, T cells and Antigen Presenting Cells) and directly inhibit their functions. Furthermore, HLA-G apparently induces differentiation of these players into cells whose functions are oriented toward active inhibition (suppressor T cells and tolerogenic Antigen Presenting Cells) (127).

Soluble HLA-G (sHLA-G) produced by trophoblasts has been detected in maternal plasma and amniotic fluid (128-130). Soluble HLA-G can act as an immunoregulatory molecule. In vitro data show that sHLA-G is able to induce apoptosis in CD8 positve NK and T cells (131-133). Furthermore, in allogeneic mixed lymphocyte cultures recombinant sHLA-G showed inhibition of cytotoxicity and induced a shift from Th1 to Th2 cytokine profile (134). Emmer et al. found a significantly lower level of amniotic fluid sHLA-G in pregnancies affected by neural tube defects as compared to controls (135). This was in concert with the findings of Seniz et al. (136). Emmer et al. suggest that sHLA-G might be involved in a mutual mechanism of on one side the association of neural tube defects and abnormalities of the thymus and on the other side the association of neural tube defects and changes in T cell repertoire.

The contribution of HLA-G to maternal-fetal tolerance and trophoblast invasion is in doubt because reports have described healthy individuals homozygous for the HLA-G*0105N allele who have been delivered normally (137;138). In the case of this allele, the full-length HLA-G1 membrane bound protein cannot be translated. Nevertheless, in HLA-G*0105N homozygous individuals, the other HLA-G2, -G3, -G6

and -G7 isoform might be produced normally, and their contribution to the immune privilege of the fetus should be taken into consideration. It has been described that, like HLA-G1, the HLA-G2, -G3 and -G4 truncated isoforms are expressed at the cell surface and modulate immune responses (139;140).

The third HLA antigen present on trophoblast is HLA-E. This is also a non-classical class I molecule with limited polymorphism. The cell-surface expression of HLA-E is dependent on its binding of signal peptides cleaved from other class I molecules. Signal peptides from both HLA-G and HLA-C have been shown to bind to HLA-E (141-143).

Maternal side

Macrophages

These extra-villous trophoblast cells at the fetal side invade the decidua of the uterus at the maternal side. In the first trimester around 40% of the cells in the decidua are leukocytes, maternal immune cells. Approximately 20% of the decidual leukocytes are macrophages. This percentage is relatively stable throughout the menstrual cycle and also in pregnancy. Strikingly, macrophages accumulate at the implantation site, while they are sparse at the site where no trophoblast invasion occurs. Interestingly, the receptor ILT2, which may recognize HLA-G, is expressed by all decidual macrophages (144). These features suggest a role for macrophages in the process of implantation and placentation.

T cells

T cells account for around 10% of the leukocytes in decidua. Unlike the macrophages, T cells do not accumulate around the implantation site. Their number remains the same throughout the menstrual cycle and early pregnancy. One mechanism that might explain the protection of the fetus from the maternal immune system is the deviation of the maternal immune system towards Th2 (T helper 2 cell) type of responses. This is called the Th1/Th2 (shift of T helper 1 cells to T helper 2 cells) paradigm. This paradigm states that established pregnancy is characterized by low levels of Th1 cytokines (Tumor Necrosis Factor-alfa (TNF-α), Interferon-gamma (IFN-γ), Interleukin-2 (IL-2)), which are known to induce abortions in a variety of animal models (145-148). Conversely,

Th2 cytokines, including IL-4 and IL-10, appear to play a protective role in pregnancy (149). However, this Th1/Th2 dichotomy now appears to be an oversimplification, because recent data on the expression of various cytokines at the fetal-maternal interface show a much more complex system (150).

Recent investigations suggest that regulatory T (Treg) cells in the decidua and peripheral blood may play an important role in protecting the fetus from alloreactive immune responses against paternal antigens at the maternal-fetal interface. Regulatory T cells are a specialized population of T cells which suppress T cell responses. The absolute number of circulating maternal Treg cells and decidual Treg cells, defined as $CD4^+CD25^+$ cells, increases progressively at each stage in human pregnancy, starting from the first trimester (151-153).

NKT cells

Natural Killer-T (NKT) cells are a small subset of T cells that also express NK-cell markers. These NKT cells play an immunomodulatory role in infection, cancer and transplantation through the production of cytokines (154). Evidence is accumulating that NKT cells might also have a role in pregnancy. The number of NKT cells is increased in decidua as compared with peripheral blood (155;156). Boyson et al. also described the expression of CD1d on extravillous trophoblast, which may form part of the NKT-cell ligand.

Natural Killer cells

NK cells, as a part of the innate immune system, are involved in the first line of defence against virally infected cells and tumour cells. They also play an important role in pregnancy. NK cells do not attack cells expressing sufficient MHC class I molecules, whereas transformed cells or cells with decreased or aberrant MHC class I expression are killed through cytolytic enzymes (157).

Phenotype

NK cells are characterized by the expression of the neural cell adhesion molecule CD56 and the concurrent absence of the T cell receptor marker CD3. Subsets of NK cells co-express the low affinity receptor CD16

(FcγRII). Based on these two surface antigens two main NK cell subsets can be distinguished. These subsets are defined by their relative expression levels of CD16 and CD56. One main subset comprises $CD56^{dim}CD16^{+}$ NK cells, that express relatively low levels of CD56 and are positive for CD16. The other subset exists of $CD56^{bright}CD16^{-}$ NK cells that express relatively high levels of CD56 and lack the marker CD16. We discovered even a third subset of NK cells, the $CD56^{superbright}$ NK cells, in the endometrium, which has not yet been described previously (158).

NK cells in peripheral blood comprise <15% of circulating lymphocytes (159). In contrast, in decidua there is a striking abundance of uterine NK cells, which constitute ~70% of resident lymphocytes (109). 90% of the peripheral NK cells are $CD56^{dim}CD16^{+}$ NK cells, whereas the majority of the uterine NK cells have the $CD56^{bright}CD16^{-}$ phenotype. The remaining 10% of peripheral NK cells also have this $CD56^{bright}CD16^{-}$ phenotype. Less than 4% of the uterine NK cells are $CD56^{dim}CD16^{+}$ (160).

Function

The two different ways of NK cell response upon activation are cytokine production and mediation of cytotoxicity. The function is depending on NK cell phenotype. The NK cell subset $CD56^{bright}CD16^{-}$ primarily produces immunoregulatory cytokines, including IFN-γ, TNF-β, LIF (Leucaemia Inhibiting Factor), IL-10, IL-13, GM-CSF (Granulocyte Macrophage-Colony-Stimulating Factor) and angiogenic growth factors (i.e. VEGF (Vascular Endothelial Growth Factor)) contributing to endometrial angiogenesis. The $CD56^{dim}CD16^{+}$ NK cell population, however, consistently produces significantly less of these cytokines. The $CD56^{dim}CD16^{+}$ NK cells mediate natural cytotoxicity and antibody-dependent cellular cytotoxicity, through the expression of both KIR (Killer cell Immunoglobulin-like Receptor) and CD16 (161-163).

So, peripheral blood NK cells differ from uterine NK cells by number, phenotype and function. Therefore uterine NK cells should be regarded as a separate lymphoid subset.

NK cell receptors

Human NK cells express both inhibitory and activating receptors that recognize different MHC class I molecules (164). As predicted by the

'missing self' hypothesis, the lack of expression of MHC molecules on target cells leads to the susceptibility of such cells to NK cell mediated lysis. These receptors play a crucial role in regulating the lytic and cytokine expression capabilities of NK cells. These NK cell receptors interact with HLA molecules expressed on the invading trophoblast (HLA-C, HLA-E and HLA-G) and thereby affect cytokine production and cytolytic activity of maternal uNK cells (165-168). It is believed that each individual NK cell and the NK cell subsets expresses a unique combination of NK cell receptors to trigger their effector functions, depending upon the nature of the target and the cytokines available in the local environment (164).

In humans three distinct families of genes have been defined that encode for receptors of HLA class I molecules. The first family identified consists of type I transmembrane molecules belonging to the immunoglobulin (Ig) superfamily and are called Killer cell Ig-like Receptors (KIR). A second group of receptors belonging to the Ig superfamily, named ILT (Immunoglobulin Like Transcripts), has more recently been described. ILTs are expressed mainly on B, T and myeloid cells, but some members of this group are also expressed on NK cells. The ligands for the KIR and some of the ILT receptors include classical HLA class I molecules, as well as the non-classical HLA-G molecule. The third family of HLA class I receptors are C-type lectin family members and are composed of heterodimers of CD94 covalently associated with a member of the NKG2 family of molecules. The ligand for most members is the non-classical class I molecule HLA-E. Each of these three families of receptors has individual members that can recognize identical or similar ligands that are signals for activation or inhibition of cellular functions. This dichotomy correlates with particular structural features present in the transmembrane and intracytoplasmic portions of these molecules. Each NK cell expresses a different combination of receptors that are present in the genome of an individual.

So far, three different receptors have been identified that can putatively interact with HLA-G, i.e., ILT2 (169;170), ILT4 (170) and KIR2DL4 (171;172). These receptors are present on the different leukocyte populations that are present in the endometrium, i.e. NK cells (KIR2DL4 and ILT2), subsets of T cells (ILT2), B-cells (ILT2 and ILT4) and monomyelocytic cells (ILT2 and ILT4). The receptors CD94/NKG2A and CD94/NKG2C recognize HLA-E on extra-villous

trophoblast. HLA-C ligands engage several members of the KIR family, i.e. KIR2DL1, KIR2DL2 and KIR2DL3 (173).

Interestingly, the different subsets of NK cells show a different NK receptor pattern and/or a different level of expression. Uterine $CD56^{bright}CD16^{-}$ NK cells express KIR at a higher level than $CD56^{dim}CD16^{+}$ NK cells (174). In contrast to blood where approximately 50% of NK cells express CD94/NKG2A, all uterine NK cells are characterized by the expression of high levels of CD94/NKG2A. We showed that the $CD56^{superbright}$ cells also differ from $CD56^{bright}$ NK cells with respect to NK cell receptor expression (158).

Interaction between NK cells and trophoblasts

Human spiral arteries are remodeled to a depth that penetrates the myometrium. This process is completed by weeks 16-20 of pregnancy (175). The muscular vessels are converted into large flaccid conduits to meet the escalating demands of the human fetus. The vascular changes are effected by extravillous trophoblast cells with the help of activated NK cells. The enlarged vessels become lined with endovascular trophoblast cells. It is believed that the spiral arteries are remodelled to a much greater depth than in other species to supply enough blood flow for the growth of the exceptionally large brain in humans (176).

As mentioned before, of the various forms of trophoblast, only the extravillous trophoblasts express MHC class I molecules, i.e. HLA-G, HLA-E and HLA-C. These molecules all have specific ligands for NK cell receptors. These ligand-receptor pairs can cause either activating or inhibitory signals. Two functions of HLA molecules on trophoblasts in modulating uterine NK cells have been postulated. First, they might protect trophoblasts from NK cell mediated lysis (177). More recent data however suggest that this protection appears to be largely independent of HLA class I expression (165;178). Therefore, the second option is more likely, which is to modulate production of cytokines and angiogenic factors by uterine NK cells in order to alter trophoblast invasion and differentiation or tissue remodeling (127;161;179). This would suggest a more finely tuned modulatory function for HLA-G, -E and –C rather than a strictly inhibitory.

Inadequate trophoblast invasion will lead to a reduced blood flow to

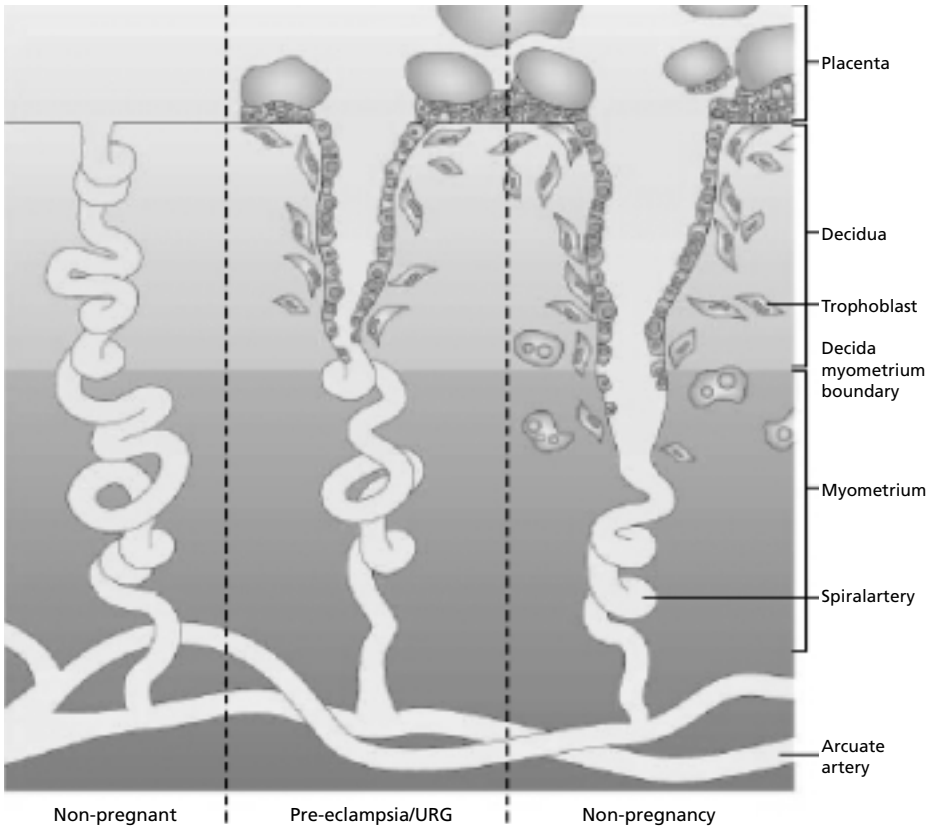


Figure 2. The blood flow through the maternal uterine arteries is increased by the infiltration of the arterial media and endothelium by extravillous trophoblast cells.
A comparison between uninvaded arteries (non-pregnant), normal pregnancy and pathological conditions of pregnancy – such as pre-eclampsia and intrauterine growth retardation (IUGR) – is shown. Note that the extent and depth of trophoblast invasion is less in pathological compared with normal pregnancy, which results in inadequate transformation of the spiral arteries in the former. This results in reduced blood flow to the feto-placental unit, which leads to poor fetal growth. The ischaemic placenta can also trigger systemic endothelial dysfunction in the mother and the onset of pre-eclampsia.
From: Nature Reviews Immunology. © Dr. A. Moffett-King (109)

the growing fetus and placenta. This can induce a variety of clinical pathological conditions such as intrauterine growth retardation, preeclampsia and miscarriage.

Evidence for an immunological role in several pregnancy disorders

IVF failure

Patients with repeated failure at IVF may include those who have an implantation–related immune abnormality. However, only limited data about this subject has been published. Beer and colleagues demonstrated in peripheral blood of idiopathic infertile women with multiple IVF failures a significantly higher level of CD56 positive NK cells as compared to fertile controls. However, they did not determine the NK cell phenotype, so it is not known whether these NK cells were mainly the $CD56^{dim}CD16^{+}$ subset (180). Fukui et al. showed a higher percentage of detrimental uterine $CD56^{dim}CD16^{+}$ NK cells in IVF patients who failed subsequent pregnancy as compared to patients with successful implantation after IVF treatment (181). A recent report of Thum et al. showed that an increase in the absolute count of activated NK cells ($NK^{dim}CD16^{+}CD69^{+}$) in peripheral blood was associated with a reduced rate of embryo implantation in IVF treatment. Furthermore, women with high peripheral $NK^{dim}CD16^{+}CD69^{+}$ cell count who were able to achieve a pregnancy had a significantly higher miscarriage rate (182).

Preeclampsia

Preeclampsia is one of the most important obstetrical diseases worldwide that causes severe maternal and perinatal morbidity and mortality. Key features of preeclampsia are maternal hypertension in combination with proteinuria. It is estimated that 6-20% of all pregnancies are affected by hypertension of which one third will develop preeclampsia (183). Women are at increased risk for preeclampsia during their first conception, and/or when the conception is with a new partner (new paternity) and when conception occurs very shortly after the beginning of their sexual relationship (short exposure to foreign semen). These features suggest an immunological role in the pathophysiology of preeclampsia.

Pregnancy related hypertension results from poor placental perfusion that is compensated for by an increase in maternal blood pressure.

In preeclampsia systemic endothelial cell dysfunction develops involving the kidneys, liver and brain. Although preeclampsia becomes symptomatic during the late second or third trimester, histological studies show an altered placental morphology that originates during early pregnancy (184;185). It is believed that preeclampsia is caused by incomplete remodeling of the spiral arteries deep into the myometrium, which leads to high maternal blood pressure and proteinuria and reduced blood flow to the fetus (Figure 2).

During preeclampsia an increased percentage and over-activity of $CD56^{dim}CD16^{+}$ NK cells are present in decidua causing disregulation of the balanced net of local interactions (186;187). The placentas of preeclamptic patients show a reduced extravillous trophoblast invasion that is associated with a lack of HLA-G expression (188-190). At the same time, recent clinical observations point to the possibility that polymorphism in both maternal and paternal genes contribute to preeclampsia. Hiby et al. showed recently that some combinations of maternal NK cell receptors and paternal HLA-C genes, which lead to the strongest inhibitory signals to NK cells, are more frequently seen in preeclampsia (191).

Endometriosis

Endometriosis affects approximately 5% to 15% of women within childbearing age (192;193). This condition is characterized by the abnormal presence of endometrial cells, mainly in the peritoneal cavity. It is most frequently associated with chronic pelvic pain, dysmenorrhea, dyspareunia and infertility. Although the pathogenesis of endometriosis remains unclear, the presence of endometrial cells in the peritoneal cavity is explained by retrograde menstruations. Because this phenomenon is prevalent in most women, several other fundamental processes must contribute to the implantation of endometrial cells and their subsequent development into endometriotic lesions. Ectopic endometrial cells must exploit strategies to escape the immune system to survive and develop into endometriotic lesions.

Although in healthy women peritoneal leukocytes eliminate ectopic endometrial cells, in patients with endometriosis, the capacity of peritoneal leukocytes to do so is significantly impaired. Activated macrophages as well as impaired cytolytic function of NK cells toward endometrial cells attributes to endometriosis (194-196). It was shown that the

inhibitory NK cell receptor KIR2DL1 was more frequently expressed by peritoneal NK cells of patients with endometriosis as compared to healthy controls. This may partly explain the decreased peritoneal NK cell activity in patients with endometriosis (197).

Recurrent miscarriage

About 12% to 15% of all clinically recognizable pregnancies result in spontaneous abortion. It is estimated that 60% of embryos are lost before clinical detection of pregnancy. Recurrent miscarriage, defined as two, three or more consecutive miscarriages, occures in 0.5-2.0% of reproducing couples (198). Fetal chromosomal abnormalities account for 50% of spontaneous abortion (199). It might be that immunological disorders account for the other half of spontaneous abortion. In women with recurrent miscarriage defective placentation was reported, in particular aberrant depth of trophoblast invasion and inadequate syncytium formation (199;200). Recent histological data on the putative association of antiphospholipid, anticardiolipin and antinuclear autoantibodies with early pregnancy loss show a relation with defective endovascular trophoblast invasion, rather than excessive intervillous thrombosis (201;202). Also women affected by the autoimmune disease systemic lupus erythematosus (SLE) are particularly prone to spontaneous abortions (198).

Women with recurrent miscarriage show a significant increase of decidual $CD56^{dim}CD16^{+}$ NK cells as compared to normal pregnancy (203). In recurrent miscarriage, the preconceptional cytotoxic reactivity of peripheral blood NK cells was increased as compared to levels found in normal pregnancy (204), although others found no difference in cytotoxicity (205). It appears that normal pregnancy is related to an increase of peripheral $CD56^{bright}$ NK cells and low peripheral cytotoxic reactivity. In contrast, increased numbers of $CD56^{dim}CD16^{+}$ NK cells and high peripheral cytotoxic reactivity seem associated with recurrent miscarriage.

Several studies have also shown a decreased expression of HLA-G in extravillous and endovascular trophoblast tissue obtained after miscarriage compared with tissue from healthy pregnancies (203;206).

Three different mechanisms have been postulated for the role of cytokines in recurrent pregnancy loss. Firstly, increased activity of uterine NK cells resulting in attack on the invading trophoblast. Secondly,

direct detrimental effects of cytokines on trophoblast cells. And thirdly, effects of cytokines on thrombotic events in the vasculature, resulting in decreased blood flow.

Aims and Outline of the Thesis

The aim of the thesis is twofold. The first aim is to develop a new IVF strategy with single embryo transfer to prevent the high number of multiple pregnancies in IVF treatment. The second aim is to gain more insight in the role of NK cell subsets in follicular fluid and endometrium during implantation in IVF treatment in order to improve the pregnancy rate eventually.

Single embryo transfer seems the only solution to minimize the multiple pregnancy rate after IVF drastically. In contrast to earlier studies we started a randomized controlled trial in which the cost-effectiveness of two cycles with SET were compared with one cycle with DET (Chapter 3). To find out whether it would be economically worthwhile to prevent the large number of multiples after IVF treatments, we first started a study in which all medical costs of IVF singleton and twin pregnancies up to 6 weeks after delivery were calculated (Chapter 2). Another approach to reduce the number of multiple pregnancies is IVF in the natural cycle, without ovarian hyperstimulation. Besides a low ongoing pregnancy rate per cycle (7.2%) (86) this method has a lot of advantages (low risks, patient-friendly, low cost). This has led to a renewed interest in natural cycle IVF. However, in case of severe oligozoospermia, ICSI in the natural cycle has never been studied serially and in a systematic way before. Therefore a pilot study of ICSI in the natural cycle was initiated (Chapter 4).

It is expected that the live birth rate after SET might be lower than after DET, especially when SET will be performed in a larger and prognostic less favourable group of patients. Therefore, it is important to improve the implantation rate of the embryo. It is believed that immunological processes play an important role during implantation, especially the interaction between maternal NK cells and HLA-G molecules on fetal trophoblast cells. When these processes are further understood it might be possible to modulate the implantation process in order to enhance the pregnancy rate. It might be possible that detrimental NK cell

subsets are associated with the cause of idiopathic infertility. We first of all hypothesized that immune cells, especially NK cells, in follicular fluid might have an influence on oocyte development and subsequent embryo quality. Consequently, in chapter 5 we studied the relation between NK cell subsets in follicular fluid and different causes of infertility. We then broadened our scope, and addressed the question whether ovarian hyperstimulation for IVF has a detrimental effect on endometrial receptivity as measured by the differential presence of NK cells subsets in the endometrium (chapter 6). In IVF, both positive and negative effects of supraphysiological levels of sex steroid hormones on pregnancy rates have been reported; in chapter 7, the functional role of NK cells and their subsets upon interaction with fetal HLA-G on trophoblast cells was further explored.

Finally, in chapter 8 the main findings of the previous chapters are summarized and discussed. In addition, the clinical implications are described and recommendations for future research are made.

Reference List

1. Beurskens MP, Maas JW, Evers JL. [Subfertility in South Limburg: calculation of incidence and appeal for specialist care]. Ned.Tijdschr.Geneeskd. 1995;139(5):235-8.
2. van Balen F, Verdurmen JE, Ketting E. Age, the desire to have a child and cumulative pregnancy rate. Hum.Reprod. 1997;12(3):623-7.
3. Kremer JA, Beekhuizen W, Bots RS, Braat DD, van Dop PA, Jansen CA et al. [Results of in vitro fertilization in the Netherlands, 1996-2000]. Ned.Tijdschr.Geneeskd. 2002;146(49):2358-63.
4. Ober WB. Reuben's mandrakes: infertility in the Bible. Int.J.Gynecol.Pathol. 1984;3(3):299-317.
5. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet 1978;2(8085):366.
6. Fishel SB, Cohen J, Fehilly C, Purdy JM, Walters DE, Edwards RG. Factors Influencing Human-Embryonic Development Invitro. Annals of the New York Academy of Sciences 1985;442(MAY):342-56.
7. Hughes GJ, Garrett DK, Lowy M. Human menopausal gonadotrophins in the treatment of unexplained infertility. Aust.N.Z.J.Obstet.Gynaecol. 1992;32(1):64-6.
8. Van Steirteghem A, Tournaye H, Van der Elst J, Verheyen G, Liebaers I, Devroey P. Intracytoplasmic sperm injection three years after the birth of the first ICSI child. Hum.Reprod. 1995;10(10):2527-8.
9. Blondel B, Macfarlane A. Rising multiple maternity rates and medical management of subfertility: better information is needed. Eur.J.Public Health 2003;13(1):83-6.
10. Braat DD, Schonbeck Y, Kremer JA. [Multiple pregnancies: epidemiology and management]. Ned.Tijdschr.Geneeskd. 2003;147(40):1952-5.
11. Benirschke K, Kim CK. Multiple pregnancy. 1. N.Engl.J.Med. 1973;288(24):1276-84.
12. Lambalk CB, de Koning CH, Braat DD. The endocrinology of dizygotic twinning in the human. Mol.Cell Endocrinol. 1998;145(1-2):97-102.
13. Templeton A, Morris JK. Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization. N.Engl.J.Med. 1998;339(9):573-7.
14. Child TJ, Barlow DH. Strategies to prevent multiple pregnancies in assisted conception programmes. Baillieres Clin.Obstet.Gynaecol. 1998;12(1):131-46.
15. Nygren KG, Andersen AN. Assisted reproductive technology in Europe,

1998. Results generated from European registers by ESHRE. European Society of Human Reproduction and Embryology. Hum.Reprod. 2001;16(11):2459-71.
16. Steegers-Theunissen. In: Bruinse HW, Visser GH, editors. Meerlingen. 1997.
17. Coetsier T, Devroey P, Dhont M, Edwards RG, Evers H, Hagglund L et al. Prevention of twin pregnancies after IVF/ICSI by single embryo transfer. Hum.Reprod. 2001;16(4):790-800.
18. Crosignani PG, Rubin BL. Multiple gestation pregnancy. Hum.Reprod. 2000;15(8):1856-64.
19. Multiple gestation pregnancy. The ESHRE Capri Workshop Group. Hum.Reprod. 2000;15(8):1856-64.
20. Buscher U, Horstkamp B, Wessel J, Chen FC, Dudenhausen JW. Frequency and significance of preterm delivery in twin pregnancies. Int.J.Gynaecol.Obstet. 2000;69(1):1-7.
21. Kinzler WL, Ananth CV, Vintzileos AM. Medical and economic effects of twin gestations. Journal of the Society for Gynecologic Investigation 2000;7(6):321-7.
22. Callahan TL, Hall JE, Ettner SL, Christiansen CL, Greene MF, Crowley WF, Jr. The economic impact of multiple-gestation pregnancies and the contribution of assisted-reproduction techniques to their incidence. N.Engl.J.Med. 1994;331(4):244-9.
23. Wolner-Hanssen P, Rydhstroem H. Cost-effectiveness analysis of in-vitro fertilization: estimated costs per successful pregnancy after transfer of one or two embryos. Hum.Reprod. 1998;13(1):88-94.
24. Senat MV, Ancel PY, Bouvier-Colle MH, Breart G. How does multiple pregnancy affect maternal mortality and morbidity? Clinical Obstetrics and Gynecology 1998;41(1):79-83.
25. Albrecht JL, Tomich PG. The maternal and neonatal outcome of triplet gestations. Am.J.Obstet.Gynecol. 1996;174(5):1551-6.
26. Douglas KA, Redman CW. Eclampsia in the United Kingdom. BMJ 1994;309(6966):1395-400.
27. Santema JG, Koppelaar I, Wallenburg HC. Hypertensive disorders in twin pregnancy. Eur.J.Obstet.Gynecol.Reprod.Biol. 1995;58(1):9-13.
28. Besinger RE. Preterm labor, premature rupture of membranes, and cervical incompetence. Curr.Opin.Obstet.Gynecol. 1993;5(1):33-9.
29. Martin, J. A., Hamilton, B. E., Ventura, S. J., Menacker, F, Park, M. M., and Sutton, P. D. Births:final data for 2001. Natl Vital Stat Rep.2002 51(2), 1-102. 2002.

30. Bergh T, Ericson A, Hillensjo T, Nygren KG, Wennerholm UB. Deliveries and children born after in-vitro fertilisation in Sweden 1982-95: a retrospective cohort study. Lancet 1999;354(9190):1579-85.
31. Scher AI, Petterson B, Blair E, Ellenberg JH, Grether JK, Haan E et al. The risk of mortality or cerebral palsy in twins: A collaborative population-based study. Pediatric Research 2002;52(5):671-81.
32. Petterson B, Nelson KB, Watson L, Stanley F. Twins, triplets, and cerebral palsy in births in Western Australia in the 1980s. BMJ 1993;307(6914):1239-43.
33. Luke B, Keith LG. The Contribution of Singletons, Twins and Triplets to Low-Birth-Weight, Infant-Mortality and Handicap in the United-States. J.Reprod.Med. 1992;37(8):661-6.
34. Ettner SL, Christiansen CL, Callahan TL, Hall JE. How low birthweight and gestational age contribute to increased inpatient costs for multiple births. Inquiry 1997;34(4):325-39.
35. Moise J, Laor A, Armon Y, Gur I, Gale R. The outcome of twin pregnancies after IVF. Hum.Reprod. 1998;13(6):1702-5.
36. Koudstaal J, Braat DD, Bruinse HW, Naaktgeboren N, Vermeiden JP, Visser GH. Obstetric outcome of singleton pregnancies after IVF: a matched control study in four Dutch university hospitals. Hum.Reprod. 2000;15(8):1819-25.
37. Koudstaal J, Bruinse HW, Helmerhorst FM, Vermeiden JP, Willemsen WN, Visser GH. Obstetric outcome of twin pregnancies after in-vitro fertilization: a matched control study in four Dutch university hospitals. Hum.Reprod. 2000;15(4):935-40.
38. Koivurova S, Hartikainen AL, Karinen L, Gissler M, Hemminki E, Martikainen H et al. The course of pregnancy and delivery and the use of maternal healthcare services after standard IVF in Northern Finland 1990-1995. Hum.Reprod. 2002;17(11):2897-903.
39. Klemetti R, Gissler M, Hemminki E. Comparison of perinatal health of children born from IVF in Finland in the early and late 1990s. Hum.Reprod. 2002;17(8):2192-8.
40. Helmerhorst FM, Perquin DA, Donker D, Keirse MJ. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. BMJ 2004;328(7434):261.
41. Jackson RA, Gibson KA, Wu YW, Croughan MS. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. Obstet.Gynecol. 2004;103(3):551-63.

42. Klentzeris LD, Bulmer JN, Warren A, Morrison L, Li TC, Cooke ID. Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. Am.J.Obstet.Gynecol. 1992;167(3):667-74.
43. Cook R, Bradley S, Golombok S. A preliminary study of parental stress and child behaviour in families with twins conceived by in-vitro fertilization. Hum.Reprod. 1998;13(11):3244-6.
44. Pinborg A, Loft A, Schmidt L, Andersen AN. Morbidity in a Danish National cohort of 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons: health-related and social implications for the children and their families. Hum.Reprod. 2003;18(6):1234-43.
45. Glazebrook C, Sheard C, Cox S, Oates M, Ndukwe G. Parenting stress in first-time mothers of twins and triplets conceived after in vitro fertilization. Fertil.Steril. 2004;81(3):505-11.
46. Gleeson C, Hay DA, Johnston CJ, Theobald TM. "Twins in school". An Australia-wide program. Acta Genet.Med.Gemellol.(Roma.) 1990;39(2):231-44.
47. Thorpe K, Golding J, Macgillivray I, Greenwood R. Comparison of Prevalence of Depression in Mothers of Twins and Mothers of Singletons. BMJ 1991;302(6781):875-8.
48. Ostfeld BM, Smith RH, Hiatt M, Hegyi T. Maternal behavior toward premature twins: implications for development. Twin.Res. 2000;3(4):234-41.
49. Mittler P. Language development in young twins: biological, genetic and social aspects. Acta Genet.Med.Gemellol.(Roma.) 1976;25:359-65.
50. Gleicher N, Campbell DP, Chan CL, Karande V, Rao R, Balin M et al. The desire for multiple births in couples with infertility problems contradicts present practice patterns. Hum.Reprod. 1995;10(5):1079-84.
51. Ryan GL, Zhang SH, Dokras A, Syrop CH, Van Voorhis BJ. The desire of infertile patients for multiple births. Fertil.Steril. 2004;81(3):500-4.
52. Nygren KG, Andersen AN. Assisted reproductive technology in Europe, 1997. Results generated from European registers by ESHRE. European IVF-Monitoring Programme (EIM), for the European Society of Human Reproduction and Embryology (ESHRE). Hum.Reprod. 2001;16(2):384-91.
53. Gardner DK, Lane M. Culture of viable human blastocysts in defined sequential serum-free media. Hum.Reprod. 1998;13 Suppl 3:148-59.
54. Behr B, Pool TB, Milki AA, Moore D, Gebhardt J, Dasig D. Preliminary clinical experience with human blastocyst development in vitro without co-culture. Hum.Reprod. 1999;14(2):454-7.

55. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. Curr.Opin.Obstet.Gynecol. 1999;11(3):307-11.
56. Huisman GJ, Fauser BC, Eijkemans MJ, Pieters MH. Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture. Fertil.Steril. 2000;73(1):117-22.
57. Coskun S, Hollanders J, Al Hassan S, Al Sufyan H, Al Mayman H, Jaroudi K. Day 5 versus day 3 embryo transfer: a controlled randomized trial. Hum.Reprod. 2000;15(9):1947-52.
58. Magli MC, Jones GM, Gras L, Gianaroli L, Korman I, Trounson AO. Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. Hum.Reprod. 2000;15(8):1781-6.
59. Munne S, Magli C, Bahce M, Fung J, Legator M, Morrison L et al. Preimplantation diagnosis of the aneuploidies most commonly found in spontaneous abortions and live births: XY, 13, 14, 15, 16, 18, 21, 22. Prenat.Diagn. 1998;18(13):1459-66.
60. Gianaroli L, Magli MC, Ferraretti AP, Munne S. Preimplantation diagnosis for aneuploidies in patients undergoing in vitro fertilization with a poor prognosis: identification of the categories for which it should be proposed. Fertil.Steril. 1999;72(5):837-44.
61. Munne S, Magli C, Cohen J, Morton P, Sadowy S, Gianaroli L et al. Positive outcome after preimplantation diagnosis of aneuploidy in human embryos. Hum.Reprod. 1999;14(9):2191-9.
62. Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G et al. Characterization of a top quality embryo, a step towards single- embryo transfer. Hum.Reprod. 1999;14(9):2345-9.
63. Vilska S, Tiitinen A, Hyden Granskog C, Hovatta O. Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. Hum.Reprod. 1999;14(9):2392-5.
64. Strandell A, Bergh C, Lundin K. Selection of patients suitable for one-embryo transfer may reduce the rate of multiple births by half without impairment of overall birth rates. Hum.Reprod. 2000;15(12):2520-5.
65. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de MM, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. Hum.Reprod. 1999;14(10):2581-7.

66. Martikainen H, Tiitinen A, Tomas C, Tapanainen J, Orava M, Tuomivaara L et al. One versus two embryo transfer after IVF and ICSI: a randomized study. Hum.Reprod. 2001;16(9):1900-3.
67. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: a prospective randomized trial. Fertil.Steril. 2004;81(3):551-5.
68. Thurin A, Hausken J, Hillensjo T, Jablonowska B, Pinborg A, Strandell A et al. Elective single-embryo transfer versus double-embryo transfer in in vitro fertilization. N.Engl.J.Med. 2004;351(23):2392-402.
69. Tiitinen A, Unkila-Kallio L, Halttunen M, Hyden-Granskog C. Impact of elective single embryo transfer on the twin pregnancy rate. Hum.Reprod. 2003;18(7):1449-53.
70. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Vercruyssen M, Barudy-Vasquez J et al. Elective single day 3 embryo transfer halves the twinning rate without decrease in the ongoing pregnancy rate of an IVF/ICSI programme. Hum.Reprod. 2002;17(10):2626-31.
71. Testart J, Lassalle B, Belaischallart J, Hazout A, Forman R, Rainhorn JD et al. High Pregnancy Rate After Early Human-Embryo Freezing. Fertil. Steril. 1986;46(2):268-72.
72. Rizk B, Smitz J. Ovarian hyperstimulation syndrome after superovulation using GnRH agonists for IVF and related procedures. Hum.Reprod. 1992;7(3):320-7.
73. Beerendonk CC, van Dop PA, Braat DD, Merkus JM. Ovarian hyperstimulation syndrome: facts and fallacies. Obstet.Gynecol.Surv. 1998;53(7):439-49.
74. Spirtas R, Kaufman SC, Alexander NJ. Fertility drugs and ovarian cancer: red alert or red herring? Fertil.Steril. 1993;59(2):291-3.
75. Rossing MA, Daling JR, Weiss NS, Moore DE, Self SG. Ovarian tumors in a cohort of infertile women. N.Engl.J.Med. 1994;331(12):771-6.
76. Bristow RE, Karlan BY. The risk of ovarian cancer after treatment for infertility. Curr.Opin.Obstet.Gynecol. 1996;8(1):32-7.
77. Duckitt K, Templeton AA. Cancer in women with infertility. Curr.Opin.Obstet.Gynecol. 1998;10(3):199-203.
78. Venn A, Watson L, Bruinsma F, Giles G, Healy D. Risk of cancer after use of fertility drugs with in-vitro fertilisation. Lancet 1999;354(9190):1586-90.
79. Klip H, Burger CW, de Kraker J, van Leeuwen FE. Risk of cancer in the offspring of women who underwent ovarian stimulation for IVF. Hum.Reprod. 2001;16(11):2451-8.

80. Paulson RJ, Sauer MV, Lobo RA. Embryo implantation after human in vitro fertilization: importance of endometrial receptivity. Fertil.Steril. 1990;53(5):870-4.
81. Chenette PE, Sauer MV, Paulson RJ. Very high serum estradiol levels are not detrimental to clinical outcome of in vitro fertilization. Fertil.Steril. 1990;54(5):858-63.
82. Simon C, Cano F, Valbuena D, Remohi J, Pellicer A. Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. Hum.Reprod. 1995;10(9):2432-7.
83. Macklon NS, Fauser BC. Impact of ovarian hyperstimulation on the luteal phase. J.Reprod.Fertil.Suppl 2000;55:101-8.
84. Daya S, Gunby J, Hughes EG, Collins JA, Sagle MA, YoungLai EV. Natural cycles for in-vitro fertilization: cost-effectiveness analysis and factors influencing outcome. Hum.Reprod. 1995;10(7):1719-24.
85. Nargund G, Waterstone J, Bland J, Philips Z, Parsons J, Campbell S. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. Hum.Reprod. 2001;16(2):259-62.
86. Pelinck MJ, Hoek A, Simons AHM, Heineman MJ. Efficacy of natural cycle IVF: a review of the literature. Hum.Reprod. Update 2002;8(2):129-39.
87. Paulson RJ, Sauer MV, Francis MM, Macaso TM, Lobo RA. In vitro fertilization in unstimulated cycles: the University of Southern California experience. Fertil.Steril. 1992;57(2):290-3.
88. Janssens RM, Lambalk CB, Vermeiden JP, Schats R, Schoemaker J. In-vitro fertilization in a spontaneous cycle: easy, cheap and realistic. Hum.Reprod. 2000;15(2):314-8.
89. Berkowitz RL, Lynch L, Chitkara U, Wilkins IA, Mehalek KE, Alvarez E. Selective reduction of multifetal pregnancies in the first trimester. N.Engl.J.Med. 1988;318(16):1043-7.
90. Evans MI, Dommergues M, Wapner RJ, Lynch L, Dumez Y, Goldberg JD et al. Efficacy of transabdominal multifetal pregnancy reduction: collaborative experience among the world's largest centers. Obstet.Gynecol. 1993;82(1):61-6.
91. Porreco RP, Harmon RJ, Murrow NS. Parental choices in grand multiple gestation: psychological considerations. J. Maternal Fetal Med. 1995;4:1111-4.
92. Brannstrom M, Enskog A. Leukocyte networks and ovulation. J.Reprod. Immunol. 2002;57(1-2):47-60.

93. Punnonen J, Heinonen PK, Teisala K, Kujansuu E, Jansen CT, Punnonen R. Demonstration of tumor necrosis factor-alpha in preovulatory follicular fluid: its association with serum 17 beta-estradiol and progesterone. Gynecol.Obstet.Invest 1992;33(2):80-4.
94. Wang LJ, Norman RJ. Concentrations of immunoreactive interleukin-1 and interleukin-2 in human preovulatory follicular fluid. Hum.Reprod. 1992;7(2):147-50.
95. Lopez BA, Newman GE, Phizackerley PJ, Laird E, Ross C, Barlow DH. Platelet-activating factor levels in human follicular and amniotic fluids. Eur.J.Obstet.Gynecol.Reprod.Biol. 1992;46(1):39-44.
96. Cataldo NA, Giudice LC. Insulin-like growth factor binding protein profiles in human ovarian follicular fluid correlate with follicular functional status. J.Clin.Endocrinol.Metab. 1992;74(4):821-9.
97. Loret dM, Jr., Flores JP, Baumgardner GP, Goldfarb JM, Gindlesperger V, Friedlander MA. Elevated interleukin-6 levels in the ovarian hyper-stimulation syndrome: ovarian immunohistochemical localization of interleukin-6 signal. Obstet.Gynecol. 1996;87(4):581-7.
98. Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum.Reprod. 1997;12(5):1047-55.
99. Bukulmez O, Arici A. Leukocytes in ovarian function. Hum.Reprod. Update 2000;6(1):1-15.
100. Hill JA, Barbieri RL, Anderson DJ. Detection of T8 (suppressor/ cytotoxic) lymphocytes in human ovarian follicular fluid. Fertil.Steril. 1987;47(1):114-7.
101. Droesch K, Fulgham DL, Liu HC, Rosenwaks Z, Alexander NJ. Distribution of T cell subsets in follicular fluid. Fertil.Steril. 1988;50(4):618-21.
102. Loukides JA, Loy RA, Edwards R, Honig J, Visintin I, Polan ML. Human follicular fluids contain tissue macrophages. J.Clin.Endocrinol.Metab 1990;71(5):1363-7.
103. Castilla JA, Sampalo A, Molina R, Samaniego F, Mozas J, Vergara F et al. Mononuclear cell subpopulations in human follicular fluid from stimulated cycles. Am.J.Reprod.Immunol. 1990;22(3-4):127-9.
104. Castilla JA, Sampalo A, Gil T, Vergara F, Jr., Molina R, Herruzo AJ.

CD8+ lymphocyte subsets in human follicular fluid. Fertil.Steril. 1992;57(5):1124-5.
105. Lachapelle MH, Hemmings R, Roy DC, Falcone T, Miron P. Flow cytometric evaluation of leukocyte subpopulations in the follicular fluids of infertile patients. Fertil.Steril. 1996;65(6):1135-40.
106. Loke YW, King A. Immunology of human placental implantation: clinical implications of our current understanding. Mol.Med.Today 1997;3(4):153-9.
107. King A, Burrows T, Verma S, Hiby S, Loke YW. Human uterine lymphocytes. Hum.Reprod.Update. 1998;4(5):480-5.
108. Santoro N, Goldsmith LT, Heller D, Illsley N, McGovern P, Molina C et al. Luteal progesterone relates to histological endometrial maturation in fertile women. J.Clin.Endocrinol.Metab. 2000;85(11):4207-11.
109. Moffett-King A. Natural killer cells and pregnancy. Nat.Rev.Immunol. 2002;2(9):656-63.
110. DeLoia JA, Stewart-Akers AM, Brekosky J, Kubik CJ. Effects of exogenous estrogen on uterine leukocyte recruitment. Fertil.Steril. 2002;77(3):548-54.
111. Medawar PB. Some Immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp.for the Soc.Exp.Biol. 1954(7):320-38.
112. Loke YW, King A, Burrows TD. Decidua in human implantation. Hum.Reprod. 1995;10 Suppl 2:14-21.
113. Zhou Y, Genbacev O, Fisher SJ. The human placenta remodels the uterus by using a combination of molecules that govern vasculogenesis or leukocyte extravasation. Ann.N.Y Acad.Sci. 2003;995:73-83.
114. Pijnenborg R, Vercruysse L, Hanssens M, van Asshe A. Incomplete trophoblast invasion: the evidence. In Critchley H, MacLean A, Poston L, Walker L (eds) Pre-eclampsia. RCOG Press, London, UK, 2003, pp. 15-26.
115. Hiby SE, King A, Sharkey A, Loke YW. Molecular studies of trophoblast HLA-G: polymorphism, isoforms, imprinting and expression in preimplantation embryo. Tissue Antigens. 1999;53(1):1-13.
116. King A, Hiby SE, Gardner L, Joseph S, Bowen JM, Verma S et al. Recognition of trophoblast HLA class I molecules by decidual NK cell receptors—a review. Placenta 2000;21 Suppl A:S81-S85.
117. Blaschitz A, Hutter H, Dohr G. HLA Class I protein expression in the human placenta. Early Pregnancy JID - 100940263 2001;5(1):67-9.

118. Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K, Marquardt H et al. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. J.Immunol. 2003;171(3):1376-84.
119. Mallet V, Fournel S, Schmitt C, Campan A, Lenfant F, Le Bouteiller P. Primary cultured human thymic epithelial cells express both membrane-bound and soluble HLA-G translated products [In Process Citation]. J.Reprod.Immunol. 1999;43(2):225-34.
120. Aractingi S, Briand N, Le Danff C, Viguier M, Bachelez H, Michel L et al. HLA-G and NK receptor are expressed in psoriatic skin: a possible pathway for regulating infiltrating T cells? Am.J.Pathol. 2001;159(1):71-7.
121. Lila N, Amrein C, Guillemain R, Chevalier P, Latremouille C, Fabiani JN et al. Human leukocyte antigen-G expression after heart transplantation is associated with a reduced incidence of rejection. Circulation 2002;105(16):1949-54.
122. Ishitani A, Geraghty DE. Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. Proc.Natl.Acad.Sci.U.S.A. 1992;89(9):3947-51.
123. Kirszenbaum M, Moreau P, Gluckman E, Dausset J, Carosella E. An alternatively spliced form of HLA-G mRNA in human trophoblasts and evidence for the presence of HLA-G transcript in adult lymphocytes. Proc.Natl.Acad.Sci.U.S.A. 1994;91(10):4209-13.
124. Fujii T, Ishitani A, Geraghty DE. A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. J.Immunol. 1994;153(12):5516-24.
125. Moreau P, Carosella E, Teyssier M, Prost S, Gluckman E, Dausset J et al. Soluble HLA-G molecule. An alternatively spliced HLA-G mRNA form candidate to encode it in peripheral blood mononuclear cells and human trophoblasts. Hum.Immunol. 1995;43(3):231-6.
126. Paul P, Cabestre FA, Ibrahim EC, Lefebvre S, Khalil-Daher I, Vazeux G et al. Identification of HLA-G7 as a new splice variant of the HLA-G mRNA and expression of soluble HLA-G5, -G6, and -G7 transcripts in human transfected cells. Hum.Immunol.JID - 8010936 2000;61(11):1138-49.
127. LeMaoult J, Le Discorde M, Rouas-Freiss N, Moreau P, Menier C, McCluskey J et al. Biology and functions of human leukocyte antigen-G in health and sickness. Tissue Antigens 2003;62(4):273-84.
128. Rebmann V, Pfeiffer K, Passler M, Ferrone S, Maier S, Weiss E et al.

Detection of soluble HLA-G molecules in plasma and amniotic fluid. Tissue Antigens. 1999;53(1):14-22.

129. Fournel S, Huc X, Aguerre-Girr M, Solier C, Legros M, Praud-Brethenou C et al. Comparative reactivity of different HLA-G monoclonal antibodies to soluble HLA-G molecules [In Process Citation]. Tissue Antigens 2000;55(6):510-8.
130. Hunt JS, Jadhav L, Chu W, Geraghty DE, Ober C. Soluble HLA-G circulates in maternal blood during pregnancy. Am.J.Obstet.Gynecol. JID - 0370476 2000;183(3):682-8.
131. Fournel S, Aguerre-Girr M, Huc X, Lenfant F, Alam A, Toubert A et al. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8+ cells by interacting with CD8. J.Immunol. 2000;164(12):6100-4.
132. Spaggiari GM, Contini P, Carosio R, Arvigo M, Ghio M, Oddone D et al. Soluble HLA class I molecules induce natural killer cell apoptosis through the engagement of CD8: evidence for a negative regulation exerted by members of the inhibitory receptor superfamily. Blood 2002;99(5):1706-14.
133. Contini P, Ghio M, Poggi A, Filaci G, Indiveri F, Ferrone S et al. Soluble HLA-A,-B,-C and -G molecules induce apoptosis in T and NK CD8+ cells and inhibit cytotoxic T cell activity through CD8 ligation. Eur.J.Immunol. 2003;33(1):125-34.
134. Kanai T, Fujii T, Kozuma S, Yamashita T, Miki A, Kikuchi A et al. Soluble HLA-G influences the release of cytokines from allogeneic peripheral blood mononuclear cells in culture. Mol.Hum.Reprod. 2001;7(2):195-200.
135. Emmer PM, Steegers EA, van Lierop MJ, Steegers-Theunissen RP, Loke YW, Joosten I. Amniotic fluid soluble human leukocyte antigen G is markedly decreased in offspring with neural tube defects. Early Hum.Dev. 2002;66(2):101-5.
136. Seniz FN, Gurakan BA, Firat S, Kayaalp A, Tokali E, Laleli Y et al. Maternal lymphocyte subsets in the cases with neural tube defects. J.Clin.Lab Immunol. 1993;40(4):181-6.
137. Ober C, Aldrich C, Rosinsky B, Robertson A, Walker MA, Willadsen S et al. HLA-G1 protein expression is not essential for fetal survival. Placenta. 1998;19(2-3):127-32.
138. Casro MJ, Morales P, Rojo-Amigo R, Martinez-Laso J, Allende L, Varela P et al. Homozygous HLA-G*0105N healthy individuals indicate

that membrane-anchored HLA-G1 molecule is not necessary for survival. Tissue Antigens 2000;56(3):232-9.

139. Menier C, Riteau B, Dausset J, Carosella ED, Rouas-Freiss N. HLA-G truncated isoforms can substitute for HLA-G1 in fetal survival. Hum. Immunol.JID - 8010936 2000;61(11):1118-25.
140. Riteau B, Rouas-Freiss N, Menier C, Paul P, Dausset J, Carosella ED. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. J.Immunol. JID - 2985117R 2001;166(8):5018-26.
141. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. Eur.J.Immunol. 1997;27(5):1164-9.
142. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J.Exp.Med. 1998;187(5):813-8.
143. Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. J.Immunol. 1998;160(10):4951-60.
144. King A, Allan DS, Bowen M, Powis SJ, Joseph S, Verma S et al. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. Eur.J.Immunol. 2000;30(6):1623-31.
145. Chaouat G, Menu E, Kinsky R, Brezin C. Immunologically mediated abortions: one or several pathways? Res.Immunol. 1990;141(2):188-95.
146. Tangri S, Raghupathy R. Expression of cytokines in placentas of mice undergoing immunologically mediated spontaneous fetal resorptions. Biol.Reprod. 1993;49(4):850-6.
147. Chaouat G, Menu E, Delage G, Moreau JF, Khrishnan L, Hui L et al. Immuno-endocrine interactions in early pregnancy. Hum.Reprod. 1995;10 Suppl 2:55-9.
148. Clark DA, Merali FS, Hoskin DW, Steel-Norwood D, Arck PC, Croitoru K et al. Decidua-associated suppressor cells in abortion-prone DBA/2-mated CBA/J mice that release bioactive transforming growth factor beta2-related immunosuppressive molecules express a bone marrow-derived natural suppressor cell marker and gamma delta T-cell receptor. Biol.Reprod. 1997;56(5):1351-60.

149. Chaouat G, Menu E, de Smedt D, Khrihnan L, Hui L, Assal MA et al. The emerging role of IL-10 in pregnancy. Am.J.Reprod.Immunol. 1996;35(4):325-9.
150. Chaouat G, Zourbas S, Ostojic S, Lappree-Delage G, Dubanchet S, Ledee N et al. A brief review of recent data on some cytokine expressions at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy. J.Reprod.Immunol. 2002;53(1-2):241-56.
151. Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. Immunology 2004;112(1):38-43.
152. Heikkinen J, Mottonen M, Alanen A, Lassila O. Phenotypic characterization of regulatory T cells in the human decidua. Clin.Exp.Immunol. 2004;136(2):373-8.
153. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol.Hum.Reprod. 2004;10(5):347-53.
154. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. Immunol.Today 2000;21(11):573-83.
155. Tsuda H, Sakai M, Michimata T, Tanebe K, Hayakawa S, Saito S. Characterization of NKT cells in human peripheral blood and decidual lymphocytes. Am.J.Reprod.Immunol. 2001;45(5):295-302.
156. Boyson JE, Rybalov B, Koopman LA, Exley M, Balk SP, Racke FK et al. CD1d and invariant NKT cells at the human maternal-fetal interface. Proc.Natl.Acad.Sci.U.S.A 2002;99(21):13741-6.
157. Pende D, Accame L, Pareti L, Mazzocchi A, Moretta A, Parmiani G et al. The susceptibility to natural killer cell-mediated lysis of HLA class I-positive melanomas reflects the expression of insufficient amounts of different HLA class I alleles. Eur.J.Immunol. 1998;28(8):2384-94.
158. van der Meer A, Lukassen HG, van Lierop MJ, Wijnands F, Mosselman S, Braat DD et al. Membrane-bound HLA-G activates proliferation and interferon-gamma production by uterine natural killer cells. Mol.Hum.Reprod. 2004;10(3):189-95.
159. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22(11):633-40.
160. Geiselhart A, Dietl J, Marzusch K, Ruck P, Ruck M, Horny HP et al. Comparative analysis of the immunophenotypes of decidual and

peripheral blood large granular lymphocytes and T cells during early human pregnancy. Am.J.Reprod.Immunol. 1995;33(4):315-22.
161. Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K et al. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. J.Clin.Endocrinol.Metab 2001;86(4):1823-34.
162. Jacobs R, Hintzen G, Kemper A, Beul K, Kempf S, Behrens G et al. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur.J.Immunol. 2001;31(10):3121-7.
163. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 2001;97(10):3146-51.
164. Lanier LL. NK cell receptors. Annu.Rev.Immunol. 1998;16:359-93.
165. King A. Uterine leukocytes and decidualization. Hum.Reprod.Update. 2000;6(1):28-36.
166. Hunt JS, Petroff MG, Burnett TG. Uterine leukocytes: key players in pregnancy. Semin.Cell Dev.Biol. 2000;11(2):127-37.
167. Kanai T, Fujii T, Unno N, Yamashita T, Hyodo H, Miki A et al. Human leukocyte antigen-G-expressing cells differently modulate the release of cytokines from mononuclear cells present in the decidua versus peripheral blood. Am.J.Reprod.Immunol. 2001;45(2):94-9.
168. Rieger L, Hofmeister V, Probe C, Dietl J, Weiss EH, Steck T et al. Th1- and Th2-like cytokine production by first trimester decidual large granular lymphocytes is influenced by HLA-G and HLA-E. Mol.Hum.Reprod. 2002;8(3):255-61.
169. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells [see comments]. J.Exp.Med. 1997;186(11):1809-18.
170. Allan DS, Colonna M, Lanier LL, Churakova TD, Abrams JS, Ellis SA et al. Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. J.Exp.Med. 1999;189(7):1149-56.
171. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. J.Exp.Med. 1999;189(7):1093-100.
172. Ponte M, Cantoni C, Biassoni R, Tradori Cappai A, Bentivoglio G, Vitale C et al. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A

and acquire p49, an HLA-G1-specific receptor [see comments]. Proc.Natl.Acad.Sci.U.S.A. 1999;96(10):5674-9.
173. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu.Rev.Immunol. 2002;20:217-51.
174. Verma S, King A, Loke YW. Expression of killer cell inhibitory receptors on human uterine natural killer cells. Eur.J.Immunol. 1997;27(4):979-83.
175. Naicker T, Khedun SM, Moodley J, Pijnenborg R. Quantitative analysis of trophoblast invasion in preeclampsia. Acta Obstet.Gynecol.Scand. 2003;82(8):722-9.
176. Robillard PY, Hulsey TC, Dekker GA, Chaouat G. Preeclampsia and human reproduction. An essay of a long term reflection. J.Reprod.Immunol. 2003;59(2):93-100.
177. Rouas Freiss N, Goncalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc.Natl.Acad.Sci.U.S.A. 1997;94(21):11520-5.
178. Avril T, Jarousseau AC, Watier H, Boucraut J, Le Bouteiller P, Bardos P et al. Trophoblast cell line resistance to NK lysis mainly involves an HLA class I-independent mechanism. J.Immunol. 1999;162(10):5902-9.
179. Trundley A, Moffett A. Human uterine leukocytes and pregnancy. Tissue Antigens 2004;63(1):1-12.
180. Beer AE, Kwak JY, Ruiz JE. Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles. Am.J.Reprod.Immunol. 1996;35(4):376-82.
181. Fukui A, Fujii S, Yamaguchi E, Kimura H, Sato S, Saito Y. Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization. Am.J.Reprod.Immunol. 1999;41(6):413-22.
182. Thum MY, Bhaskaran S, Abdalla HI, Ford B, Sumar N, Shehata H et al. An increase in the absolute count of CD56dimCD16+CD69+ NK cells in the peripheral blood is associated with a poorer IVF treatment and pregnancy outcome. Hum.Reprod. 2004;19(10):2395-400.
183. Robillard PY. Interest in preeclampsia for researchers in reproduction. J.Reprod.Immunol. 2002;53(1-2):279-87.
184. De Wolf F, Brosens I, Robertson WB. Ultrastructure of uteroplacental arteries. Contrib.Gynecol.Obstet. 1982;9:86-99.
185. Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, van Asshe A. A study of placental bed spiral arteries and trophoblast invasion in

normal and severe pre-eclamptic pregnancies. Br.J.Obstet.Gynaecol. 1994;101(8):669-74.
186. Dekker GA, Sibai BM. The immunology of preeclampsia. Semin.Perinatol. 1999;23(1):24-33.
187. Wilczynski JR, Banasik M, Tchorzewski H, Glowacka E, Malinowski A, Szpakowski M et al. Expression of intercellular adhesion molecule-1 on the surface of peripheral blood and decidual lymphocytes of women with pregnancy-induced hypertension. Eur.J.Obstet.Gynecol.Reprod.Biol. 2002;102(1):15-20.
188. Hara N, Fujii T, Yamashita T, Kozuma S, Okai T, Taketani Y. Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia: immunohistological demonstration with anti-HLA-G specific antibody "87G" and anti- cytokeratin antibody "CAM5.2". Am.J.Reprod.Immunol. 1996;36(6):349-58.
189. Lim KH, Zhou Y, Janatpour M, McMaster M, Bass K, Chun SH et al. Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. Am.J.Pathol. 1997;151(6):1809-18.
190. Goldman-Wohl DS, Ariel I, Greenfield C, Hochner-Celnikier D, Cross J, Fisher S et al. Lack of human leukocyte antigen-G expression in extravillous trophoblasts is associated with pre-eclampsia. Mol.Hum.Reprod. 2000;6(1):88-95.
191. Hiby SE, Walker JJ, O'shaughnessy KM, Redman CW, Carrington M, Trowsdale J et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. J.Exp.Med. 2004;200(8):957-65.
192. Houston DE, Noller KL, Melton LJ, III, Selwyn BJ, Hardy RJ. Incidence of pelvic endometriosis in Rochester, Minnesota, 1970-1979. Am.J.Epidemiol. 1987;125(6):959-69.
193. Eskenazi B, Warner ML. Epidemiology of endometriosis. Obstet.Gynecol.Clin.North Am. 1997;24(2):235-58.
194. Oosterlynck DJ, Meuleman C, Waer M, Vandeputte M, Koninckx PR. The natural killer activity of peritoneal fluid lymphocytes is decreased in women with endometriosis. Fertil.Steril. 1992;58(2):290-5.
195. Braun DP, Gebel H, House R, Rana N, Dmowski NP. Spontaneous and induced synthesis of cytokines by peripheral blood monocytes in patients with endometriosis. Fertil.Steril. 1996;65(6):1125-9.
196. Wu MY, Yang JH, Chao KH, Hwang JL, Yang YS, Ho HN. Increase in the expression of killer cell inhibitory receptors on peritoneal

natural killer cells in women with endometriosis. Fertil.Steril. 2000;74(6):1187-91.
197. Maeda N, Izumiya C, Yamamoto Y, Oguri H, Kusume T, Fukaya T. Increased killer inhibitory receptor KIR2DL1 expression among natural killer cells in women with pelvic endometriosis. Fertil.Steril. 2002;77(2):297-302.
198. Regan L. Recurrent early pregnancy failure. Curr.Opin.Obstet.Gynecol. 1992;4(2):220-8.
199. Pellicer A, Rubio C, Vidal F, Minguez Y, Gimenez C, Egozcue J et al. In vitro fertilization plus preimplantation genetic diagnosis in patients with recurrent miscarriage: an analysis of chromosome abnormalities in human preimplantation embryos. Fertil.Steril. 1999;71(6):1033-9.
200. Michel MZ, Khong TY, Clark DA, Beard RW. A morphological and immunological study of human placental bed biopsies in miscarriage. Br.J.Obstet.Gynaecol. 1990;97(11):984-8.
201. Stern C, Chamley L, Hale L, Kloss M, Speirs A, Baker HW. Antibodies to beta2 glycoprotein I are associated with in vitro fertilization implantation failure as well as recurrent miscarriage: results of a prevalence study. Fertil.Steril. 1998;70(5):938-44.
202. Sebire NJ, Fox H, Backos M, Rai R, Paterson C, Regan L. Defective endovascular trophoblast invasion in primary antiphospholipid antibody syndrome-associated early pregnancy failure. Hum.Reprod. 2002;17(4):1067-71.
203. Emmer PM, Steegers EA, Kerstens HM, Bulten J, Nelen WL, Boer K et al. Altered phenotype of HLA-G expressing trophoblast and decidual natural killer cells in pathological pregnancies. Hum.Reprod. 2002;17(4):1072-80.
204. Aoki K, Kajiura S, Matsumoto Y, Ogasawara M, Okada S, Yagami Y et al. Preconceptional natural-killer-cell activity as a predictor of miscarriage. Lancet 1995;345(8961):1340-2.
205. Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veerhoek M, Joosten I. Peripheral Natural Killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. Hum.Reprod. 2000;15(5):1163-9.
206. Fan L, Zhang X, Xu L, Yang J, Li W, Liu B. Preliminary study on the expression of HLA-G mRNA in normal placenta and placenta with RSA after immunotherapy. Transplant Proc 1999;31(4):1854-6.

PART I

Clinical Aspects

2

Cost analysis of singleton versus twin pregnancies after in vitro fertilization

Marieke H.G.M. Lukassen[1]
Yvonne Schönbeck[1]
Eddy M.M. Adang[2]
Didi D.M. Braat[1]
Gerhard A. Zielhuis[3]
Jan A.M. Kremer[1]

Departments of Obstetrics and Gynaecology[1], Medical Technology Assessment [2] and Epidemiology and Biostatistics[3], University Medical Center Nijmegen, Nijmegen, the Netherlands.

Fertility and Sterility 2004; 81:1240-1246

Abstract

STUDY OBJECTIVE:
Determine the difference in cost between singleton and twin pregnancies after IVF-treatment from pregnancy until six weeks after delivery from health care perspective.

DESIGN:
Retrospective cost analysis.

SETTING:
IVF department at the University Medical Center Nijmegen (UMCN), the Netherlands.

PATIENTS:
A representative sample of singleton and twin pregnancies after IVF-treatment between 1995-2001 at the UMCN.

INTERVENTION:
IVF, with or without ICSI and with or without cryo-preservation.

MAIN OUTCOME MEASURE:
Medical cost per singleton and twin pregnancy after IVF.

RESULTS:
In patients with twin pregnancies the incidence of hospital antenatal care, complicated vaginal deliveries and cesarean sections was higher and they were associated with more frequent and longer maternal and neonatal hospital admissions. Maternal and neonatal hospital admissions were the major cost-drivers. The medical cost per twin pregnancy was found to be more than five times higher than per singleton pregnancy, 13,469 euro and 2,550 euro respectively.

CONCLUSIONS:
The medical cost per twin pregnancy was more than 10,000 euro higher than per singleton pregnancy. Reduction of the number of twin pregnancies by elective single embryo transfer (SET) will save substantial amounts of money. This might be used for additional IVF-cycles, which are probably needed to achieve similar success rates with SET as with two embryo transfer.

Introduction

Since the introduction of in-vitro fertilization (IVF) 25 years ago, the number of multiple pregnancies has increased dramatically (1-3). Nowadays, most clinics in Europe transfer two embryos per cycle to reduce chances for high-order multiple pregnancies (4). In the Netherlands a maximum of two embryos are routinely transferred per cycle. However, the risk for a multiple pregnancy after IVF-treatment is still 25% (5), which is a 15 fold increase relative to the risk of 1.6% after natural conception (6).

Multiple pregnancies have been shown to require more medical care than singleton pregnancies. Multiple pregnancies are associated with higher risks for hypertension, pre-eclampsia, intrauterine growth retardation, prematurity, low birth weight and cesarean section (6-8). In addition, perinatal morbidity and mortality are increased as compared to singletons (6-8). Moreover, IVF-pregnancies (singletons and multiples) are at greater risk for obstetric and perinatal complications than spontaneously conceived pregnancies (9-12). Due to these effects medical costs are expected to be higher for multiple pregnancies than for singleton pregnancies (8;13;14).

In previous studies, age of the woman, number of previous cycles and quality of embryos available for transfer were identified as the most important predictors for multiple birth (15-17). Single Embryo Transfer (SET) in women with good prognosis for pregnancy after IVF-treatment would be a successful method to reduce the number of twin pregnancies. However, whether SET is cost-effective needs to be determined, since pregnancy rates probably decrease inversely proportional to the number of embryos transferred. Even in women with a good prognosis more cycles might be needed to realize the same success percentage with SET as with two embryos transfer (18). Prevention of twin pregnancies by SET will reduce twin pregnancy related complications and the associated medical expenses. The question is whether these savings will pay for the expenses of the extra IVF-cycles required for SET.

The aim of this study was to determine the differences in costs between singleton and twin pregnancies after IVF-treatment from pregnancy until six weeks after delivery from health care perspective.

Materials and methods

For this study, cost drivers from pregnancy until six weeks (42 days) after delivery were determined for singleton and twin pregnancies. Estimates of volumes were based on data from a representative sample of twin and singleton pregnancies from the IVF-population at the University Medical Center Nijmegen (UMCN). Unit costs were based on real costs where possible, the remaining unit costs were based on hospital charges. Total costs per singleton pregnancy and twin pregnancy were calculated and compared. The protocol was approved by the Medical Ethical Review Committee of our hospital.

Medical process

Antenatal care during the first weeks of pregnancy was routinely performed by a gynecologist at the hospital where the couple received IVF-treatment. According to the Dutch system of antenatal care, patients with low risk singleton pregnancies were advised to seek antenatal care provided by a midwife. If medical complications arose during pregnancy, antenatal care was taken over by a gynecologist or midwife under supervision of a gynecologist at a hospital. Patients with singleton pregnancies who preferred hospital antenatal care, patients who had medically complicated pregnancies and all patients with twin pregnancies received hospital antenatal care by a gynecologist or midwife under supervision of a gynecologist at a hospital.

Delivery for patients with medically uncomplicated pregnancies took place either at home or at the outpatient department of a hospital. Delivery for patients with medically complicated pregnancies took place at the hospital as inpatient. Inpatient deliveries included at least one day of maternal hospital stay. Cesarean sections were always followed by 3-10 days of hospital stay, depending on maternal and neonatal condition and on hospital regulations. In addition, due to complications during pregnancy or delivery and in the six weeks after delivery, maternal and neonatal hospital admissions occurred.

This description led to the following cost-drivers: mode of antenatal care, mode of delivery and maternal and neonatal hospital admission days.

Estimation of volumes

Estimations of volumes were based on data from a representative sample of twin and singleton pregnancies from a database containing all couples with a live born singleton or at least one live born twin after IVF treatment (IVF/ICSI/cryo-IVF/cryo-ICSI) at the UMCN between 1995-2001 (n=963 pregnancies, 24% twins). From the database the 172 most recent twin pregnancies and 168 singleton pregnancies with a similar date of embryo transfer were selected.

To obtain data not included in the database regarding the course of pregnancy as well as the occurrence of maternal and neonatal hospital admissions, all subjects were sent a questionnaire. The questionnaire contained questions about antenatal care, mode of delivery, days of maternal hospital admission from pregnancy until six weeks after delivery and days of neonatal hospital admission during the first six weeks (maximum of 42 days) after birth. In addition, participants were asked for their consent to verify uncertain or missing data in medical records.

Antenatal care was categorized as either antenatal care by a midwife, provided outside the hospital or 'hospital antenatal care', provided by a gynecologist or midwife under supervision of a gynecologist at a hospital. In case of referral during pregnancy from antenatal care by a midwife to hospital antenatal care, only the latter was charged.

Deliveries were categorized as home delivery, uncomplicated vaginal delivery (spontaneous birth of singletons), complicated vaginal delivery (vacuum and forceps extractions and twin deliveries) and cesarean section. For twin deliveries in which the first child was born by vaginal delivery and the second by cesarean section, both deliveries were charged separately. This might cause the sum of twin deliveries to be greater than the number of twin pregnancies.

No distinction between various hospital departments was made for maternal hospital admission. Neonatal hospital admission was divided in admission to a Neonatal Intensive Care Unit (NICU) and non-NICU departments. Since admission to a NICU is much more expensive than admission to non-NICU departments, all NICU admissions mentioned in the questionnaires, including length of stay, were verified in medical records to make sure a non-NICU department was not mistaken for a NICU.

Data on method of conception (IVF/ICSI/cryo-IVF/cryo-ICSI), maternal age, birth weight and gestational age were obtained from the

Table 1. Characteristics of the study population per pregnancy, unless otherwise indicated.

	Singleton pregnancies (n=135)	Twin pregnancies (n=144)
Mode of conception		
IVF (%)	66 (48.9)	68 (47.2)
ICSI (%)	63 (46.7)	68 (47.2)
Cryo-IVF (%)	6 (4.4)	6 (4.2)
Cryo-ICSI (%)	0	2 (1.4)
Gender (% male) [a]	67 (50.0)	132 (46.0)
Maternal age (years) [b]	34.5 ± 3.4	33.2 ± 3.6
Gestational age (weeks) [a, b]	39.6 ± 1.7	36.5 ± 2.5
Birth weight (grams) [a, b]	3,341 ± 540	2,425 ± 586

[a] Calculated per newborn.

[b] Values are means ± SD.

Note: ICSI = intracytoplasmic sperm injection.

existing database. Gestational age for pregnancies after IVF-treatment was defined as 14 days before the puncture of the oocytes until birth. For pregnancies after cryo-IVF and cryo-ICSI-treatment gestational age was defined as 17 days before the date of embryo-transfer until birth.

Estimation of unit costs

Unit costs for NICU admission were obtained from real-cost-calculations of the Dutch National Health Tariffs Authority (CTG) (CTG policy rule nr. I-612; personal communication). Unit costs for admission to a non-NICU department and for maternal hospital admission were based on real-cost-calculations as described by Oostenbrink *et al.* (19). National price indexes were used to convert real-costs to 2002 rates. Unit costs of antenatal care and delivery were based on hospital charges, since real costs were not available and since they were not expected to be major cost-drivers. Hospital charges for the year 2003 were obtained from the CTG (20-22). All unit costs were presented in euro's.

Statistical Analyses

Data were presented as percentages (number (N)) and means ± standard deviation (SD) or [range]. The Mann-Whitney-U test was performed on mean total cost per singleton and twin pregnancy. All analyses were done in Statistical Package for the Social Sciences (SPSS) version 10.0 for Windows. Sensitivity analyses were applied to major cost-drivers of

Table 2. Data on antenatal care and delivery per pregnancy

	Singleton pregnancies (n=135)	Twin pregnancies (n=144)
Antenatal care (%)		
Midwife	28 (20.7)	0
Hospital (gynaecologist/midwife)	107 (79.3)	144 (100.0)
Delivery (%)		
Vaginal uncomplicated	88 (65.2) [a]	0
Vaginal complicated	20 (14.8)	87 (60.4)
Cesarean section	27 (20.0)	57 (39.6)
Maternal admission (%)	116 (85.9)	144 (100.0)
Length of stay [b] (days)	3.6 (0-50)	10.1 (1-59)
NICU admission [c] (%)	3 (2.2)	36 (12.5)
Length of stay [c, b] (days)	0.11 (0-11)	1.8 (0-42) [d]
Non-NICU admission [c] (%)	39 (28.9)	189 (65.6)
Length of stay [c, b] (days)	2.0 (0-33)	8.8 (0-42) [d]

[a] Including 13% (N=18) home deliveries.

[b] Values are means (range).

[c] Calculated per newborn.

[d] Six weeks (42 days) was maximum length of follow-up.

Note: NICU = neonatal intensive care unit.

which available prices were uncertain. Sensitivity analyses were performed in Microsoft Excel 2000.

Results

Study Population

In total, 279 pregnancies were included in this study (response rate: 82%) of which 135 were singleton pregnancies and 144 were twin pregnancies. Table 1 summarizes the characteristics of the study population. There were no differences in the methods of conception or gender between singleton and twin pregnancies. Maternal age at delivery, gestational age and birth weight were all substantially lower in twin pregnancies than in singleton pregnancies. The number of babies born with a low birth weight (<2500 gram) or a very low birth weight (<1500 gram) was significantly higher among twins than among singletons (51% versus 3% and 5.9% versus 1.5%, respectively, p<0.001). The number of preterm births

(gestational age <37 weeks) was not significantly different between the singletons and twins (32% and 36%, respectively). This was also the case for the number of births below 32 weeks of gestational age between singletons and twins (2.2% and 3.8%, respectively).

The non-response rate was 16% (n=28) in the twin pregnancy group and 19% (n=31) in the singleton pregnancy group. The maternal age, gestational age, birth weight and gender were not different between the responders and non-responders. The methods of conception in the twin pregnancy group did also not differ between the responders and non-responders. In the singleton pregnancy group, however, a higher percentage of the non-responders became pregnant by cryo-IVF and cryo-ICSI as compared with the responders (29.1% and 4.4%, respectively, $p<0.05$). This difference might be based on coincidence due to small numbers.

Quantities and Costs

Data on antenatal care, delivery and hospital admission days are presented in Table 2. In the study population all patients with twin pregnancies and 79% of the patients with singleton pregnancies received hospital antenatal care. Of this last group, five percent initially received antenatal care by a midwife, but was referred during pregnancy. The remaining 21% of patients with singleton pregnancies received antenatal care by a midwife. Unit cost of total antenatal care by a midwife was 283 euro. Unit cost of total hospital antenatal care was 88 euro. The unit cost of antenatal care was based on charges. Due to the fact that overhead expenses from an out-patient clinic can be distributed by more gynecologists and patients than in a midwife practice, the charges for antenatal care by a gynecologist is much lower. As antenatal care by a midwife occurred only in singletons, total cost of antenatal care per singleton pregnancy was substantially higher than per twin pregnancy: (20.7% x 283 euro + 79.3% x 88 euro) = 128 euro versus 88 euro.

As defined before, all vaginal twin deliveries (60%) were regarded as complicated. Of the singleton pregnancies 65% was an uncomplicated vaginal delivery, including 13% home deliveries. Singleton vaginal deliveries were in 15% complicated. The cesarean section rate for patients with twin pregnancies was 40%, in contrast to 20% in singleton pregnancies. Since six twin deliveries were vaginal for the first born, but cesarean section for the second born, a total of 150 deliveries were charged. The

Table 3. Cost (in euros) per singleton and twin pregnancy after IVF.

	Singleton pregnancy	Twin pregnancy	Difference
Antenatal care	128	88	-40
Delivery costs	553	700	187
Hospital care mother	1,113	3,147	2,034
Neonatal care (including NICU)	755	9,534	8,779
Total costs	2,549	13,469	10,920

unit costs of vaginal deliveries were 489 euro for an uncomplicated and 512 euro for a complicated vaginal delivery. Home deliveries were priced at 309 euro. The unit cost of a cesarean section was 912 euro. Total cost per twin delivery was (90 x 512 euro + 60 x 912 euro) / 144 = 700 euro and (13% x 309 euro + 52% x 489 euro + 14.8% x 512 euro + 20% x 912 euro) = 553 euro per singleton delivery.

All patients with twin pregnancies after IVF-treatment were admitted to a hospital for at least one day from pregnancy until six weeks after delivery, while 86% of the patients with singleton pregnancies was admitted. The mean duration of hospital stay was 10.1 days (SD = 10.5) for patients with twin pregnancies and 3.6 days (SD = 6.1) for patients with singleton pregnancies. The unit cost for a hospital admission day was 313 euro. Total cost for maternal hospitalization per patient with a twin pregnancy was 3,147 euro and 1,113 euro per patient with a singleton pregnancy.

Of the newborns from twin pregnancies 66% was admitted to a hospital after birth, compared to 29% of the newborns from singleton pregnancies. Admission to a NICU during the first six weeks after birth occurred in 12% of the newborns from twin pregnancies and in 2% of the singletons. Newborns from twin pregnancies, on average, stayed longer at a NICU than those from singleton pregnancies (1.8 days [SD = 6.8] and 0.11 days [SD = 0.98], respectively). Admission to non-NICU departments during the first six weeks after birth occurred in 65% of the newborns from twin pregnancies and 29% of the newborns from singleton pregnancies (including newborns transferred from a NICU). The average length of stay at these departments for newborns from twin pregnancies was substantially higher than for newborns from singleton pregnancies (8.8 days [SD = 10.6] and 2.0 days [SD = 5.2], respectively). The unit cost for admission at a NICU was 1,121 euro per day. The unit cost for

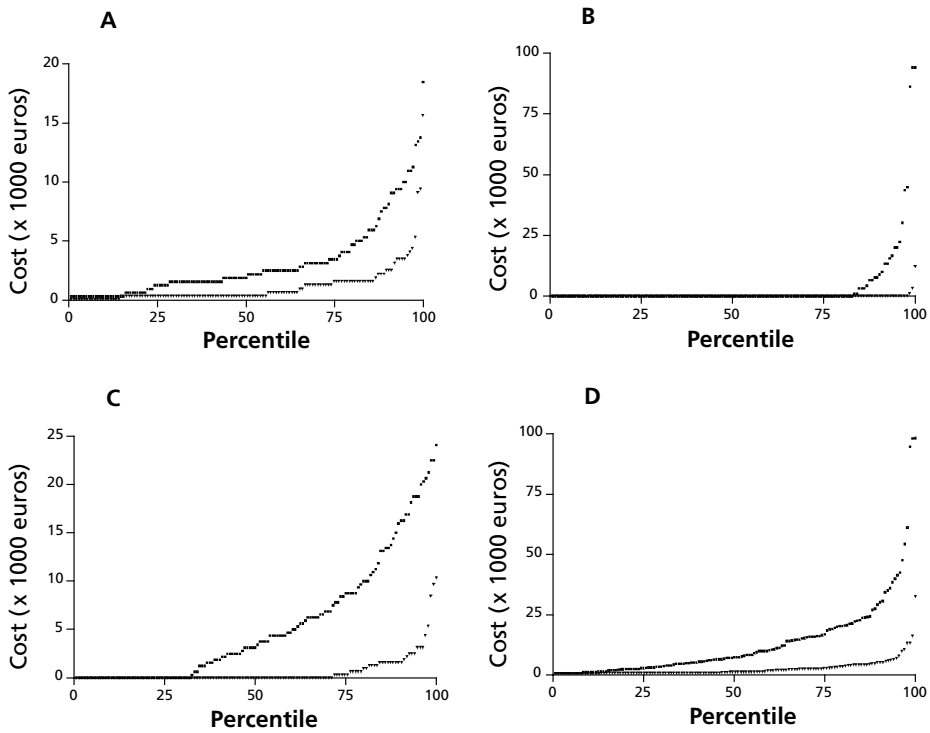


Figure 1.
Major cost-drivers and total cost per singleton (triangles) and twin (squares) pregnancy. Each triangle (singleton) and square (twin) represents the cost of a pregnancy per patient with respect to **A**, the maternal admission cost, or the cost of a live born baby with respect to **B**, the neonatal intensive care unit (NICU) cost, **C**, the non-NICU cost, or **D**, the total cost. The number of pregnancies or live-born babies were shown as percentile.

admission at a non-NICU department was 313 euro. Total cost of neonatal hospital admission per twin was [(1.8 x 1.121 euro + 8.8 x 313euro)] x 2 = 9,534 euro and (0.11 x 1.121 euro + 2.0 x 313 euro) = 755 euro per singleton.

In Figure 1 cost of the major cost-drivers (A: maternal admissions, B: NICU-admissions, C: non-NICU-admissions) and the total cost per pregnancy (D) are presented for singleton and twin pregnancies. In Table 3 the costs of singleton pregnancies, twin pregnancies and the differences are summarized. Costs were indexed on 2002 rates. Mean total cost per twin pregnancy was significantly higher than per singleton pregnancy after IVF (Mann-Whitney-U test: $p<0.001$), causing a more than 10,000 euro difference in costs.

Sensitivity Analysis

One-way sensitivity analysis for the cost per neonatal admission day at a non-NICU department was performed, because for this study no distinction between various non-NICU departments was made. Since there will be a difference in costs for admission for observation only, admission to Medium-Care (MC) and High-Care (HC), the unit cost for non-NICU admission was varied from 177 euro (minimum cost per admission day in a regional hospital; 19) to 510 euro (cost per HC admission day; CTG policy rule I-612; personal communication). This caused the mean difference between singleton and twin pregnancies to range from 8,803 euro to 13,985 euro.

One-way sensitivity analysis for the cost per maternal admission day was performed, since for this study no distinction between various hospital departments for maternal hospital admission was made. The unit cost was varied from 177 euro (minimum price per admission day in regional hospital; 19) to 458 euro (maximum price per admission day at a university medical center; 19). This caused the mean difference between singleton and twin pregnancies to range from 10,036 euro to 11,862 euro.

When these sensitivity analyses were combined, the mean difference between singleton and twin pregnancies varied from 7,919 euro to 14,928 euro.

Discussion

The results of this study show that the medical costs from induction of IVF-pregnancy until six weeks after delivery per twin pregnancy were more than five times higher than per singleton pregnancy (13,469 euro and 2,550 euro, respectively). This difference in costs of more than 10,000 euro was mainly caused by an increased number and duration of maternal and neonatal hospital admissions. Even though NICU days were by far the most expensive hospital days in this study, due to the shortage of room at NICU-departments, newborns were referred to non-NICU-departments as soon as possible, causing non-NICU-departments to be the major cost-driver.

Sensitivity analyses for hospital days (the major cost drivers) did not largely affect the difference in cost between twin and singleton pregnancies. Since all major cost drivers occurred more frequently in twin preg-

nancies than in singleton pregnancies, twin pregnancies would have turned out to be more expensive at any chosen unit cost.

In this study we focused on the costs of pregnancies with a subsequent live born baby. Costs of pregnancies resulting into stillbirth were not calculated. Five percent (n=12) of all twin pregnancies (n=240) and 2% (n=16) of all singleton pregnancies (n=751) after IVF between 1995 and 2001 resulted into stillbirth. This difference was statistically significant ($p<0.05$), therefore we expect that the twin pregnancies will even be more expensive as compared to the singleton pregnancies. For this study we decided to present means (including mean costs) for both normally distributed and skewed data. It is well known that high medical expenses occur only in a few pregnancies, causing a skewed distribution of costs. Logistic transformation would largely diminish the effect of these expensive pregnancies, thereby leading to a major underestimation of the costs that could actually be saved by prevention of twin pregnancies. To illustrate this: geometric means (95% confidence interval [CI]) of the total costs for twin and singleton pregnancies were 7,247 euro (95% CI: 5,990 euro – 8,769 euro) and 1,618 euro (95% CI: 1,401 euro – 1,867 euro) respectively (Student t-test: $p<0.001$).

Previous studies on costs of singleton and twin pregnancies were largely hypothetical, not based on data from IVF-pregnancies and/or included rough estimates of prices (13;14;18). Since Klemetti et al. (10) found that the outcome for IVF infants was poorer than those for other infants, we used data from IVF-pregnancies only. In addition, unit costs were based on real cost prices as much as possible. For some minor cost drivers of which real cost prices were not available, hospital charges were used. This might have caused the actual costs to vary slightly.

Comparing the results from our study with previous studies was difficult, since medical practice, definitions and prices vary widely between and even within countries. Furthermore, from some cost-effectiveness studies, it was not possible to extract data on cost per pregnancy. The costs per pregnancy found in our study were lower than those found in a study by Wolner-Hanssen and Rydhstroem (13). However, their study was performed from a societal perspective and therefore included costs of sick leave and (medical and societal) costs of handicap. Leaving these costs out caused the costs per singleton pregnancy to be similar (2,550 euro vs. 2,821 euro). The major discrepancy in cost per twin pregnancy between their study and ours was found in the cost of neonatal care. This

difference could partly be explained by the fact that in their study, in contrast to the Dutch situation, both children from a twin were admitted to a NICU for an equally long period, even if only one required NICU treatment. This caused the neonatal care costs in their study to be much higher than in this study.

In our study no data on handicaps were included, because only costs made from pregnancy until six weeks after delivery were counted. Since handicaps occur more often in children born premature and since twins are more often born premature than singletons (7;8;23), adding medical costs for handicaps would have increased the difference in costs between twin and singleton pregnancies on the longer term (13), indicating that the 10,000 euro difference in cost between twin and singleton pregnancies is an underestimation of the actual difference in cost on the longer term.

Antenatal care was classified as antenatal care by a midwife or hospital antenatal care, but not classified according to intensity of use. Previous Finnish studies found women with twin pregnancies after IVF to use more specialized antenatal care and use this care more intensively (9;10). Including classification of intensity of antenatal care use would cause the cost of antenatal care per twin pregnancy to increase.

Recall errors due to the fact that some women were pregnant several years ago, might have occurred in the number and duration of reported maternal and neonatal hospital admissions. However, since twin and singleton pregnancies were matched on date of embryo transfer, recall errors will probably have occurred equally in both groups. Therefore, recall bias was unlikely to be an issue in the estimation of the difference in costs between singleton and twin pregnancies.

Although twin pregnancies are associated with a higher complication rate and associated costs, there might be one financial argument in favor of a twin pregnancy: the birth of a twin might reduce the desire for an additional pregnancy for some couples. In this selective population of IVF-couples a twin pregnancy might save the costs of additional IVF-treatments and costs related to pregnancy and delivery to achieve a second pregnancy and subsequent live-born baby. Depending on the costs of achieving pregnancy by SET, two children born from two singleton pregnancies after SET might be more expensive than a twin. However, we think that, due to costs for handicap on the longer term, even in this situation twin pregnancies are not likely to be in favor of singleton pregnancies in terms of costs.

This study focussed on costs for twin and singleton pregnancies from the medical point of view. Savings on the medical budget due to reduction of the number of twin pregnancies by SET might be spent on other medical treatments. In contrast, savings in other compartments, such as society (sick leave, travel expenses), are not likely to become available for medical treatments and were therefore not included in this study (24). The money that can be saved by reduction of the number of twin pregnancies by SET might be used for additional IVF-cycles needed to achieve similar success rates with SET as with two embryo transfer. An effectiveness study for SET is needed, to determine whether SET is cost-effective.

References

1. Toner JP. Progress we can be proud of: U.S. trends in assisted reproduction over the first 20 years. Fertil.Steril. 2002;78:943-50.
2. Nygren KG, Andersen AN. Assisted reproductive technology in Europe, 1997. Results generated from European registers by ESHRE. European IVF-Monitoring Programme (EIM), for the European Society of Human Reproduction and Embryology (ESHRE). Hum.Reprod. 2001;16:384-91.
3. Steegers-Theunissen RP, Zwertbroek WM, Huisjes AJ, Kanhai HH, Bruinse HW, Merkus HM. Multiple birth prevalence in The Netherlands. Impact of maternal age and assisted reproductive techniques. J.Reprod. Med. 1998;43:173-9.
4. Templeton A, Morris JK. Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization. N.Engl.J.Med. 1998;339:573-7.
5. Nygren KG, Andersen AN. Assisted reproductive technology in Europe, 1999. Results generated from European registers by ESHRE. Hum.Reprod. 2002;17:3260-74.
6. Multiple gestation pregnancy. The ESHRE Capri Workshop Group. Hum. Reprod. 2000;15:1856-64.
7. Buscher U, Horstkamp B, Wessel J, Chen FC, Dudenhausen JW. Frequency and significance of preterm delivery in twin pregnancies. Int.J.Gynaecol. Obstet. 2000;69:1-7.
8. Kinzler WL, Ananth CV, Vintzileos AM. Medical and economic effects of twin gestations. J.Soc.Gynecol.Investig. 2000;7:321-7.
9. Koivurova S, Hartikainen AL, Karinen L, Gissler M, Hemminki E, Martikainen H et al. The course of pregnancy and delivery and the use of maternal healthcare services after standard IVF in Northern Finland 1990-1995. Hum.Reprod. 2002;17:2897-903.
10. Klemetti R, Gissler M, Hemminki E. Comparison of perinatal health of children born from IVF in Finland in the early and late 1990s. Hum.Reprod. 2002;17:2192-8.
11. Koudstaal J, Braat DD, Bruinse HW, Naaktgeboren N, Vermeiden JP, Visser GH. Obstetric outcome of singleton pregnancies after IVF: a matched control study in four Dutch university hospitals. Hum.Reprod. 2000;15:1819-25.
12. Koudstaal J, Bruinse HW, Helmerhorst FM, Vermeiden JP, Willemsen WN, Visser GH. Obstetric outcome of twin pregnancies after in-vitro fertilization: a matched control study in four Dutch university hospitals. Hum.Reprod. 2000;15:935-40.

13. Wolner-Hanssen P, Rydhstroem H. Cost-effectiveness analysis of in-vitro fertilization: estimated costs per successful pregnancy after transfer of one or two embryos. Hum.Reprod. 1998;13:88-94.
14. Callahan TL, Hall JE, Ettner SL, Christiansen CL, Greene MF, Crowley WF, Jr. The economic impact of multiple-gestation pregnancies and the contribution of assisted-reproduction techniques to their incidence. N.Engl.J.Med. 1994;331:224-49.
15. Martikainen H, Tiitinen A, Tomas C, Tapanainen J, Orava M, Tuomivaara L et al. One versus two embryo transfer after IVF and ICSI: a randomized study. Hum.Reprod. 2001;16(9):1900-3.
16. Strandell A, Bergh C, Lundin K. Selection of patients suitable for one-embryo transfer may reduce the rate of multiple births by half without impairment of overall birth rates. Hum.Reprod. 2000;15:2520-5.
17. Vilska S, Tiitinen A, Hyden-Granskog C, Hovatta O. Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. Hum.Reprod. 1999;14:2392-5.
18. De Sutter P, Gerris J, Dhont M. A health-economic decision-analytic model comparing double with single embryo transfer in IVF/ICSI. Hum.Reprod. 2002;17:2891-6.
19. Oostenbrink JB, Koopmanschap MA, Rutten FFH. Guidelines for cost analyses. Methods and guidelines for economic evaluations in health care [Dutch]. Amstelveen: College voor Zorgverzekeringen, 2000.
20. Dutch National Health Tariffs Authority. Tariffs for midwives 2003. Utrecht: COTG, 2002.
21. Dutch National Health Tariffs Authority. Tariffs for medical specialists 2003 (excluding psychiatrists). Utrecht: COTG, 2002.
22. Dutch National Health Tariffs Authority. Tariffs for institutions 2003. Utrecht: COTG, 2002.
23. Bergh T, Ericson A, Hillensjo T, Nygren KG, Wennerholm UB. Deliveries and children born after in-vitro fertilisation in Sweden 1982-95: a retrospective cohort study. Lancet 1999;354:1579-85.
24. Adang EMM. Debatable Efficiency [Dutch]. Economisch Statistische Berichten. 4349 ed.,2002:170-1.

3

Two cycles with single embryo transfer versus one cycle with double embryo transfer: a randomized controlled trial

Marieke H.G.M. Lukassen[1]
Didi D.M. Braat[1]
Alex M.M. Wetzels[1]
Gerhard A. Zielhuis[2]
Eddy M.M. Adang[3]
Eduard Scheenjes[4]
Jan A.M. Kremer[1]

Departments of Obstetrics and Gynecology[1], Epidemiology and Biostatistics[2] and Medical Technology Assessment[3], University Medical Centre Nijmegen, Nijmegen, the Netherlands.

Department of Obstetrics and Gynecology[4], Gelderse Vallei Ziekenhuis, Ede, the Netherlands.

Human Reproduction 2005; 20:702-708

Abstract

BACKGROUND:
To reduce the number of multiple pregnancies after IVF we investigated the effectiveness of two cycles with single embryo transfer (SET) and one cycle with double embryo transfer (DET) after IVF and calculated the cost-effectiveness of both strategies.

METHODS:
A randomized controlled trial was performed in 107 women, aged < 35 years, in their first IVF cycle, with at least one good quality embryo. They were randomized to the SET (n = 54) or DET (n = 53) group using a computer-generated random block number table, stratified for primary or secondary infertility.

RESULTS:
The cumulative live birth rates per woman randomized of two consecutive cycles of SET [41%, 95% confidence interval (CI): 27-54] versus one cycle of DET (36%, 95% CI 23-49) were comparable, whereas the multiple pregnancy rate rate was significantly higher: 37% (95% CI 15-59) in the DET and 0% in the in the SET group (p = 0.002). Combining the medical costs of the IVF-treatments (where 1.5 more SET cycles were required to achieve each live birth) and of pregnancies up to six weeks after delivery, the total medical costs of DET per live birth were 13,680 euro and 13,438 euro for SET.

CONCLUSIONS:
Two cycles with SET are equally effective as one cycle with DET, and the medical costs per live birth up to six weeks after delivery are the same. However, if lifetime costs for severe handicaps are included, more than 7,000 euro per live birth will be saved after implementing SET. Because of the high probability of multiple pregnancies in this group of IVF-patients, only SET should be performed.

Introduction

Today multiple pregnancies are considered to be the most serious complication of IVF treatment for both mother and child. In the Netherlands a maximum of two embryos are routinely transferred per cycle to prevent higher-order multiple pregnancies. The risk for a twin pregnancy with this regime, however, is still 20-35% (1), which is a 15-fold increase relative to the risk of 1.6% after natural conception (1;2).

There is a need to convince both health care workers and the infertile couples that multiple pregnancies are not a desirable outcome of an IVF-treatment. The maternal mortality in Europe is twice as high for multiple pregnancies as compared with singleton pregnancies (3). Multiple pregnancies are associated with higher risks to hypertensive disorders, anaemia and haemorrhage during pregnancy (4). The risk of neonatal death in twins is even seven times that of singletons (5). In infants from multiple pregnancies many perinatal complications are attributable to the fact that they are more likely to be born prematurely and with a lower birth weight than babies from singleton pregnancies (6). There is an increased risk of long-term medical and developmental problems, in particular neurological impairment, in children from multiple pregnancies. Therefore, there is a higher risk of severe handicap in twins (7). Multiple pregnancies impose also a steep burden on government expenses and health services (8-11).

Retrospective studies identified age, number of embryos available and quality of embryos as the most important predictors for multiple birth (12;13).

The only solution to minimize twin pregnancies after IVF is to transfer one embryo per cycle. Up to now only four randomized controlled trials comparing single embryo transfer (SET) and double embryo transfer (DET) have been published (14). The study of Gerris et al. was performed in 53 patients less than 34 years of age and had at least two top quality embryos available. They found an ongoing pregnancy rate of 38.5% in the SET group (38.5%) and 74.1% in the DET group (14). The study of Martikainen et al. was performed in 144 patients with at least four good quality embryos. There was no statistical difference in cumulative live birth rate (fresh and frozen cycles) between the SET group (39%) and the DET group (51%) (15). Gardner et al. randomized 48 IVF patients with at least 10 follicles > 12 mm on day of HCG administration

to either transfer of one blastocyst or two blastocysts on day 5. There was no significant difference in ongoing pregnancy rate between single blastocyst transfer (61%) and double blastocyst transfer (76%) (16). Finally, Thurin et al. performed a multi-centre randomized trial in 661 patients less than 36 years of age having at least two good quality embryos available. The cumulative ongoing pregnancy rate in the SET group (one fresh SET and one frozen SET cycle) was 40% versus 44% in the DET group (without a frozen DET cycle; p = 0.344) (17). Only an abstract of this study has been published. While these randomized controlled trials make an important contribution to the SET discussion, the number of subjects are relatively small, except for the study of Thurin et al., and the studies were not combined with a cost analysis to determine the cost-effectiveness of both strategies.

Therefore, we conducted an additional randomized controlled trial, in which we compared the live birth rate after two consecutive SET cycles with the live birth rate after one DET cycle. Freeze-thaw cycles were not included in this study. We hypothesized that two SET cycles might be needed to compensate for the possibly lower pregnancy rate as compared with DET. This study was performed among patients with a high risk for multiples (women less than 35 years of age, first IVF treatment cycle and at least two embryos of which one of excellent or good quality). Additionally, we calculated the cost-effectiveness of both strategies to determine the best transfer strategy for efficiency decisions. The SET strategy could be more expensive due to the extra SET cycles needed to achieve an equal live birth rate, while the DET strategy could generate more costs because of the complications related to twin pregnancies. The cost analysis was done by implementing the results of our previous retrospective study (18), showing that the medical costs of twin pregnancies were more than five times higher than the costs of singleton pregnancies after IVF.

Materials and methods

Study design

The protocol was approved by the Medical Ethical Review Committee of the University Medical Centre Nijmegen and all couples participating in the study signed a written informed consent after they had been thor-

oughly informed regarding the strict study design. The study objective was to investigate the live birth rate of SET after two consecutive treatment cycles with the live birth rate of DET after one treatment cycle, excluding freeze-thaw cycles. Cumulative live birth rate was the primary outcome measure. Multiple births, live birth rates after only one treatment cycle and clinical pregnancy rates (number of abortions and ectopic pregnancies) were secondary outcome measures.

Participants

Only patients undergoing their first IVF/ICSI cycle ever or the first cycle after a successful treatment were included. The age of the women had to be younger than 35 years (at the time of ET) with a basal follicle-stimulating hormone (FSH) level <10 IU/L (12;13). Patients with a medical reason for elective SET were excluded, e.g. uterine malformation or history of cervical incompetence. At least two embryos with one excellent (grade 4) or one good (grade 3) quality embryo had to be available for transfer on day 3 after oocyte retrieval during the first cycle, according to Steer *et al.* (19): grade 4: no fragmentation; grade 3: <10% fragmentation; grade 2: 10-50% fragmentation; grade 1: >50% of the embryo is fragmented. The number of blastomeres was not an inclusion criterion. A total of 494 IVF patients from the department of Obstetrics and Gynecology at the University Medical Centre in Nijmegen and Gelderse Vallei Hospital in Ede underwent oocyte retrieval and embryo transfer in the University Medical Centre in Nijmegen and were younger than 35 years (figure 1). The pregnant patients were followed up until delivery. Of the 494 IVF patients, 217 did not agree to participate or were excluded because of the following reasons: request for DET in the supposition that they would obtain the highest chance of pregnancy after the first cycle, request for SET to avoid multiple pregnancy, having concerns about randomisation as such, and basal FSH level ≥ 10 IU/L. From 162 patients the number or quality of the available embryos did not meet the inclusion criteria and eight patients had a medical reason for SET. The characteristics of the patients and their treatment cycles are shown in table I.

Clinical follow-up

A total of 107 patients were randomized to the SET (n = 54) or DET group (n = 53) from January 2001, to February 2003. All pregnant patients were followed up until delivery. The characteristics of the ran-

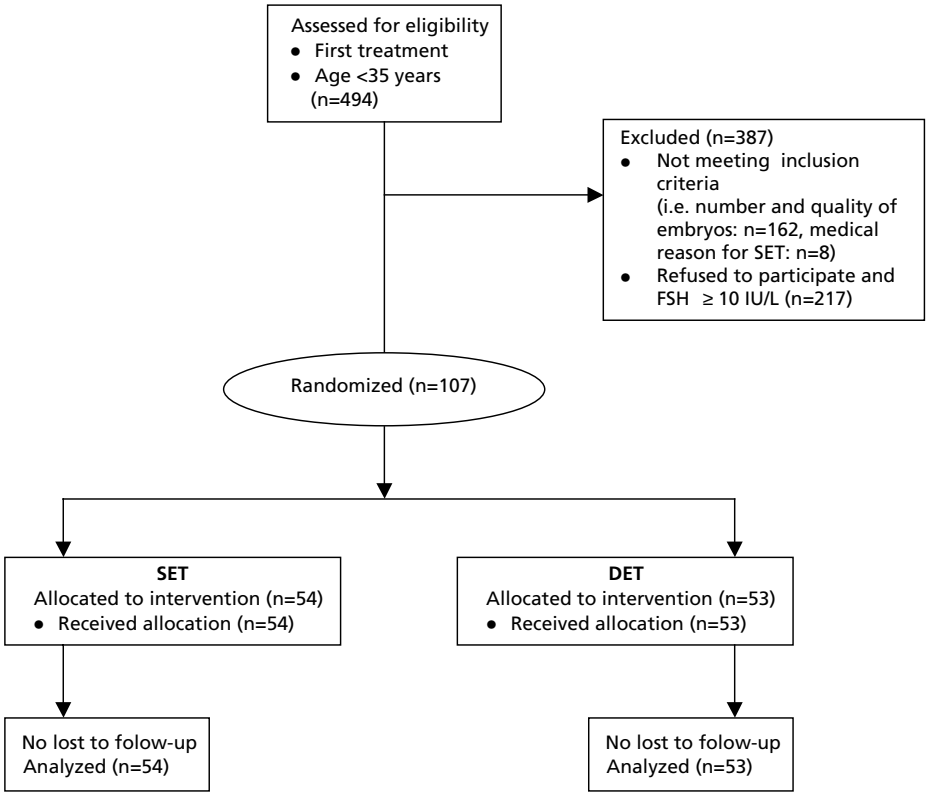


Figure 1. Trial profile.

domized patients and their first treatment cycle were similar between the SET and DET group, as well as between the participants and non-participants. ICSI was performed in 28/54 cycles (52%; 95% CI 38-66) of the SET, 21/53 (40%; 95% CI 26-53) of the DET and 117/217 (54%; 95% CI 47-61) of the cycles of the non-participants. These percentages were not statistically different. The number of patients with at least one good quality embryo available for transfer was not different between the SET and DET group (Table I).

All patients completed the first treatment cycle. One patient in the SET group experienced total fertilization failure in the second treatment cycle. Four patients in the SET group did not (yet) undergo their second treatment cycle. One patient became pregnant spontaneously and one patient got divorced after the first treatment cycle. Two patients still have to undergo the second cycle. These patients were analyzed as if no preg-

Table I. Baseline characteristics of the study subjects and the non-participants of the first cycle

Variable	SET (*n* = 54)	DET (*n* = 53)	non-participants (*n* = 217)
Age (years) [mean (SD; range)]	30.2 (3.2; 20-34)	31.2 (2.9; 25-34)	30.6 (3.0; 20-34)
Duration of infertility (months) [mean (SD; range)]	7 (17; 6-99)	42 (23; 7-123)	41.2 (23.3; 5.7-139.5)
Primary infertility [*n* (%)]	40 (74)	38 (72)	162 (72)
Basal FSH level (IU/l)	6.6 (1.5)	6.6 (1.8)	NA
Etiology of infertility [*n* (%)]			
Male factor	36 (67)	26 (49)	140 (64)
Tubal	5 (9)	9 (17)	24 (11)
Unexplained	5 (9)	14 (27)	32 (15)
Other female	8 (15)	4 (8)	21 (10)
ICSI [*n* (%)]	28 (52)	21 (40)	117 (54)
Oocytes [mean (SD; range)]	13.4 (6.1; 3-29)	12.6 (6.4; 3-26)	11.8 (5.7; 2-43)
Embryos [mean (SD; range)]	8.0 (4.1; 2-18)	7.8 (4.1; 2-18)	7.0 (4.0; 2-24)
$\geq$ 1 good embryo [*n* (%)]	40 (74)	35 (66)	127 (59)
Cryopreserved [mean (SD; range)]	1.8 (2.0; 0-8)	0.9 (1.8; 0-8)	

NA = not available.

nancy had occurred. In the SET group two patients elected to receive two instead of one embryo at transfer in the second cycle. One of these patients became pregnant with a singleton. Three patients unintentionally received two embryos during the second SET cycle by mistake. Violation of the protocol after randomization occurred in two cases: in the SET group in one patient no embryo transfer took place because of severe ovarian hyperstimulation syndrome. The embryos of this cycle were cryopreserved and one good quality embryo was transferred after thawing, resulting in an abortion. In the DET group one patient was included despite the fact that this patient already underwent a previous IVF-treatment cycle with transfer of only one moderate quality embryo. No pregnancy occurred. In the current cycle two good quality embryos were transferred resulting in a singleton pregnancy. Because of the intention-to-treat principle all patients mentioned above were included in the analyses.

Assignment

Randomization to the SET or DET group was performed using a computer-generated random block number table, stratified for primary or secondary infertility, executed by an independent statistician. Allocation to the randomized group by an opaque, sealed envelope took place just before embryo transfer by the laboratory personnel to maintain concealment to the last moment. Both patients and physicians were not blinded to treatment group.

IVF/ICSI procedure

Pituitary desensitization (long protocol) was achieved using triptoreline (Decapeptyl®; Ferring, the Netherlands). Ovarian stimulation was accomplished by recombinant follicle-stimulating hormone (Puregon®; Organon, Oss, the Netherlands). Thirty-six hours after the injection of 10.000 IU of human chorionic gonadotrophin (hCG) (Pregnyl®; Organon, Oss, the Netherlands) we performed transvaginal oocyte retrieval. Embryo transfer was performed 3 days after oocyte retrieval.

Luteal support was given through progestogen intravaginal capsules of 100 mg, at a daily dose of 600 mg (Progestan®; Organon, Oss, the Netherlands). On day 15 after embryo transfer a pregnancy test was performed, using a commercial urinary kit. Five weeks after embryo transfer clinical pregnancy was confirmed by ultrasonic evidence of an intrauterine gestational sac and a positive heart beat.

After the retrieval, oocyte-cumulus complexes were prepared, washed and incubated (37°C, 5% CO_2 in air) in IVF medium, consisting of human tubal fluid medium (20) (Bio Whittaker, Verviers, Belgium) supplemented with 10% pasteurized plasma solution (Central Laboritory of Bloodtransfusion, Amsterdam, the Netherlands). Freshly ejaculated semen was mixed with 5 ml IVF medium, layered onto an 80% Pure-Sperm (Nidacon International, Gothenberg, Sweden) gradient, centrifuged at 600g for 20 minutes and washed twice with IVF medium. Insemination was carried out in Falcon dishes by adding about 200,000 motile spermatozoa to the oocytes in 2 ml of IVF medium. If ICSI was performed, the oocytes were treated with hyaluronidase solution (Medi-cult, Jyllinge, Denmark) and denuded with a capillary pipette before injection was performed. ICSI was performed according to the method described by Van Steirteghem and colleagues (21). Only morphologically normal (at 200x magnification), motile spermatozoa were injected. After injection or

insemination, the oocytes were transferred to 50 ml drops of culture mineral oil (1 oocyte/droplet), and were judged for the presence of 0, 1, 2 or ≥3 pronucleï (pn). On day 3 after oocyte retrieval, the embryos were scored for cell number and embryo quality score on a scale from 1 to 4 according to Steer *et al.* (19).

Excess embryos of good morphological quality (< 10% fragmentation and at least 7 blastomeres) were cryopreserved using the standard protocol with the cryoprotectant 1,2-propanediol (22).

Cost-effectiveness analysis of singleton versus twin pregnancies after IVF

The cost-effectiveness analyses of both embryo transfer strategies were performed by using the results of a previous study of our group (18) and by using the direct medical costs of an IVF cycle in the Netherlands [Dutch National Health Tariffs Authority (CTG)].

In the previous study we determined the medical costs from pregnancy after IVF up to six weeks after delivery, using the following cost-drivers: mode of antenatal care, mode of delivery and maternal and neonatal hospital admission days (18). Estimations of volumes were based on data from a representative sample of twin (n = 172) and singleton (n = 168) pregnancies from a database containing all couples with a live born singleton or at least one live born twin after IVF treatment at the UMCN between 1995-2001 (n = 963 pregnancies; 24% twins). The medical costs from the induction of IVF-pregnancy up to six weeks after delivery were 13,469 euro for twin pregnancies and 2,550 euro for singleton pregnancies; $p < 0.001$) (18).

Furthermore, the direct medical costs of an IVF treatment in the Netherlands, including hospital charges and medication costs of a long protocol and a mean starting dose of 200 IU recombinant FSH, have been researched using 2003 rates [Dutch National Health Tariffs Authority (CTG)]. The medical costs of freeze-thaw cycles were not included. This cost analysis was combined with the randomized controlled trial data using the primary outcome measure 'live birth' for effectiveness of treatment. This results in a cost-effectiveness ratio using a medical perspective of costs per live birth rate.

Statistical analysis

The analyses were based upon the intention-to-treat principle. Cumulative live births were analyzed as primary outcome (first plus second fresh

cycle in SET group). Multiple births, live birth rate after just one IVF cycle in the SET group, ongoing pregnancy rate and number of abortions and extra-uterine pregnancies were analyzed as secondary outcome. Categorical data were analyzed by the χ^2-test. The Student's *t*-test for independent samples was used to test continuous variables between the SET and double embryo transfer group.

Power analysis indicated a requirement of 52 patients in each group to show a reduction in live birth rate of 20% in the SET group after one cycle (assuming a live birth rate of 50% in the double embryo transfer group and 30% in the SET group after one cycle {[1 - (1 - 0.3)^2] x 100 = 51% after two cycles)} with an α error of 0.10 and a β error of 0.20, tested one-sided.

P-values ≤ 0.05 were considered significant. All analyses were performed two-sided by SPSS (Statistics Package for Social Sciences).

Results

Clinical results

The primary outcome, the cumulative live birth rate between the two strategies, was in the same order of magnitude between two cycles of SET (22/54; 41%, 95% CI 27-54) and the DET group (19/53; 36%, 95% CI 23-49) (Table II). So, the difference in live birth rate between two fresh cycles of SET and one fresh cycle of DET is 5% (95% CI –10.5-20.5). The number of twins, as secondary outcome, in the DET group was seven (6 twins and 1 dizygotic triplet) out of 19 live births (37%; 95% CI 15- 59) versus 0 out of 21 in the SET group (p = 0.002). One fetus of a twin pregnancy was stillborn at 36 weeks of gestation, cause unknown. The cumulative incidence of miscarriages and ectopic pregnancies was also similar in the SET and DET group, respectively 8/54 (15%; 95% CI 5-25) and 6/53 (11%; 95% CI 3-20).

The number of live children born in the DET group was 26, including one dizygotic triplet and one stillborn child. The number of preterm born babies was not significantly higher in the DET group than in the SET group, respectively 5/25 (20%; 95% CI 4-36) and 2/22 (9%; 95% CI –3-21). Nine out of ten babies born before 37 weeks of gestation were from twin pregnancies. The number of babies with a low birth weight (< 2500 gram) was higher in the DET group, entirely attributable to the

Table II. The cumulative outcome of fresh embryo transfers

Variable	SET (*n* = 54)			DET (*n* = 53)	*P*
	1st cycle	2nd cycle	Cumulative		
No. of subjects	54	40	54	53	NS
No. of transfers	54	35[a]	89	53	NS
Clinical pregnancy [*n* (%)]	20 (37)	10 (25)	30 (56)	25 (47)	NS
Miscarriage [*n* (%)]	6 (11)	2 (5)	8 (15)	5 (9)	NS
Ectopic pregnancy [*n* (%)]	0	0	0	1 (2)	NS
Live birth [*n* (%)]	14 (26)	8 (20)	22 (41)	19 (36)	NS
Singleton [*n* (%) of live births]	14 (100)	8 (100)	22 (100)	12 (63)	NS
Twin ([*n* (%) of live births]	0	0	0	7[b] (37)	0.002
Perinatal death (*n*)	0	0	0	1[c,d]	NS
Preterm birth < 37 weeks [*n* (%)]	2 (14)	0	2[e] (10)	5[d] (20)	NS
Low birthweight infants (<2500 g) [*n* (%)]	1 (7)	0	1[e] (5)	10[d] (40)	0.002

Data given are mean (%).

[a] In 5 patients no embryo transfer took place: one patient got divorced, one became pregnant spontaneously, one experienced total fertilization failure, two still have to undergo the second cycle.

[b] One dizygotic triplet.

[c] Prenatal death: one fetus from a twin pregnancy died *in utero* at 36 weeks amenorrhoe.

[d] Number of live born children in DET group = 26

[e] Number of live born children in SET group = 22

NS = not significant.

twins, as compared with the SET group, respectively 10/26 (38%; 95% CI 19- 58) and 1/22 (5%; 95% CI −4-13, p = 0.002).

Even if we compared just one cycle of SET with DET, the live birth rate was not significantly different between the two groups, respectively 14/54 (26%; 95% CI 14-38) and 19/53 (36%; 95% CI 23-49; Table II). The difference in live birth rate between one cycle of SET and DET is -10% (95% CI −24.7-4.7). The percentages of miscarriages and ectopic pregnancies were similar in both groups, 6/54 (11%; 95% CI 3-20) after one cycle in the SET and 6/53 (11%; 95% CI 3-20) in the DET group.

Health economic results

The cost-effectiveness starts with the calculation of the costs of the IVF-treatments per live birth. The calculated costs are all expressed per live birth, although the outcome of a live birth can be one healthy or sick child

or two, even three, healthy and/or sick children. So the mean costs per live birth are calculated. The medical costs of an IVF-treatment in the Netherlands are 2,532 euro, including the medication [2003, Dutch National Health Tariffs Authority (CTG)].

The mean number of IVF-cycles performed per live birth was 4.3 in the SET group [(54 + 40 cycles) / 22 live births] and 2.8 in the DET group (53 cycles / 19 live births). So 1.5 more SET cycles were needed per live birth (4.3/2.8). The medical costs of the IVF-treatment per live birth in the SET group are 10,888 euro (4.3 x 2,532 euro) and 7,090 euro in the DET group (2.8 x 2,532 euro). This results in extra treatment costs per live birth after SET of 3,798 euro (10,888 euro – 7,090 euro). The percentage of live born twins was 0% in the SET group and 37% in the DET group. Based on the results of a previous study (18) the average medical costs of an IVF-pregnancy up to six weeks after delivery were 13,469 euro for twin pregnancies and 2,550 euro for singleton pregnancies. For the DET group this resulted in 6,590 euro per live birth (37% x 13,469 euro + 63% x 2,550 euro), compared with 2,550 euro for SET (100% x 2,550 euro). Medical costs of the pregnancies up to 6 weeks after delivery after SET were 4,040 euro (6,590 euro – 2,550 euro) lower than after DET.

Combining the medical costs of the IVF-treatments and pregnancies up to 6 weeks after delivery, the total medical costs of SET per live birth are 13,438 euro (10,888 euro + 2,550 euro) and 13,680 euro (7,090 euro + 6,590 euro) for DET.

Discussion

In order to diminish the number of twin pregnancies after IVF substantially, single embryo transfer is considered a serious option for daily IVF-practice. Before such protocols can be implemented, the effectiveness of SET had to be determined. Our randomized controlled trial assessed the live birth rate after two consecutive IVF-treatment cycles utilising single embryo transfer compared with one cycle with double embryo transfer, in a population with a high risk for multiple births. The cumulative live birth rate in the SET group (22/54; 41%, 95% CI 27-54) was remarkably similar to the live birth rate in the DET group (19/53; 36%, 95% CI 23-49). The difference in live birth rate between two cycles of SET and DET is therefore 5% (95% CI –10.5-20.5). In contrast, six twins and one triplet

were born out of 19 live births in the DET group (37%, 95% CI 15-59) versus zero twins out of 22 live births in the SET group (p = 0.002). Even after one treatment cycle the live birth rate in the SET group (14/54; 26%, 95% CI 14-38) was already close to, and not significantly different from the rate in the DET group (19/53; 36%, 95% CI 23-49). The difference in live birth rate between one cycle of SET and DET is -10% (95% CI −24.7-4.7).

The characteristics of the patients and first treatment cycle were not different between the SET and DET group (Table I), indicating that the randomization was successful. As the pregnancy rate in the non-response group (103/276; 37%) was similar to the rate in the DET group (19/53; 36%) selection bias has not likely occurred.

To date, four randomized controlled trials comparing one versus two embryo transfer have been performed (14;15). Gerris et al. performed their study in a highly selected population. Patients were included with at least two top quality embryos (4 or 5 blastomeres on day two, at least 7 blastomeres on day three, <20% fragments and the absence of multinucleated blastomeres). This may explain the high pregnancy rates in their SET (38.5%) and DET group (74.1%). At the same time, the number of twin pregnancies in the non-eligible population was also remarkably high (35.8%). So, it can be expected that the effect of SET in this highly selected group will have little effect on the overall multiple pregnancy rate. However, Gerris and colleagues studied in a subsequent retrospective cohort analysis over a four year period the effect of elective SET in a larger group of patients (n = 1559). They showed that at least one top embryo with the embryo criteria described above could be transferred in ~70% of all cycles. Over these four years SET increased from 13% to 31%, whereas the ongoing pregnancy rate per retrieval did not change (35.9% to 31.0%), but the multiple pregnancy rate decreased with almost 50% (33.6% to 18.6%) (23). Martikainen and colleagues used the following inclusion criteria for the embryo quality: at least four good quality embryos available (even sized blastomeres and <20% fragmentation on day 2), but remarkably almost half of the patients fulfilled the inclusion criteria, so SET was applicable to a large group of patients. Gardner et al. evaluated all blastocysts using a previously described scoring system (24), but they did not describe inclusion criteria for blastocyst quality in their randomized controlled trial. Thurin et al. included only patients with at least two good quality embryos available, but the definition for

good quality was not described in their abstract. In our study only one good quality embryo out of a total of at least two embryos had to be available with less than 10% fragmentation, irrespective of the number of blastomeres, so SET could be performed in a large group of patients. The present study makes a contribution to properly designed randomized controlled trials evaluating the effectiveness of SET in a selected IVF population. In the future performing a meta-analysis could raise the level of evidence with respect to the effectiveness of SET. This study is distinguished from the other studies by the fact that we performed two consecutive fresh cycles with SET versus one cycle with DET to determine how many extra cycles were needed to obtain a similar live birth rate per strategy.

Another notable difference with the other randomized controlled trials is that we also performed a cost-effectiveness analysis. We used the medical costs from the induction of IVF-pregnancy up to six weeks after delivery for singleton and twin pregnancies, determined in a previous published retrospective study by our group (18). The total costs per live birth of the two strategies in the present study, including IVF-treatments and pregnancy up to 6 weeks after delivery, did not differ significantly (SET: 13,438 euro, DET: 13,680 euro). Gerris et al. just recently showed in a real-life prospective health economic study that in Belgium SET cost 7,126 euro and DET 11,039 euro. All medical costs from IVF-treatment, pregnancy and neonatal period up to 3 months after delivery were prospectively analyzed in patients of <38 years of age. One cycle of SET with one high quality embryo resulting in a live birth rate of 37.4% was compared with one cycle of DET resulting in a live birth rate of 36.6%. In the DET group embryo quality was not a selection criterion, therefore both groups were not comparable with respect to chance of pregnancy. This was not a randomized controlled trial, but based upon patient choice for SET or DET, whereas SET was exclusively performed if a high quality embryo was available (11).

In our study however, the long term outcome of the DET strategy may be much more expensive when adding lifetime costs for handicaps. A French study from 1982 showed that the risk of serious handicap was 6.9-fold higher for a twin child as compared with a singleton child (25). Based on these figures, Wolner-Hanssen calculated that the estimated average cost for care of severely handicapped children was 20,477 euro for twins and 1,489 euro for singletons (average euro value of SEK in

2004 = 9.219) (9). So, in the DET group the lifetime costs for handicaps will be 8,514 euro (37% x 20,477 euro + 63% x 1,489 euro). In the SET group this amount will be 1,489 euro (100% x 1,489 euro). Finally, combining the costs of IVF-treatments and (singleton and twin) IVF-pregnancies up to six weeks after delivery with the costs for severely handicapped (singleton and twin) children, DET live births will cost 22,194 euro (13,680 euro + 8,514 euro) and SET live births 14,927 euro (13,438 euro + 1,489 euro). So, hypothetically when handicaps are included, the DET strategy could be more than 7,000 euro more expensive per live birth than the SET strategy.

An additional advantage of SET is the higher number of cryopreserved embryos, which can contribute to the pregnancy rate per cycle. The mean number of cryopreserved embryos per first treatment cycle was 1.8 (SD 2.0; range 0-8) in the SET group and 0.9 (SD 1.8; range 0-8) in the DET group). Unfortunately, the frozen-thawed embryo transfers did not contribute to the overall live birth rate in our study. Two Finnish studies reported an increase of the pregnancy rate due to cryopreserved embryos in patients treated with elective SET of 18% (13) to 26% (26). Martikainen and colleagues observed an increase in live birth rate from 30% to 39% after frozen-thawed embryo transfers in the SET group (15). It seems worthwhile to put effort in improving the cryopreservation programme, which will reduce the number of second cycles with fresh SET and as a consequence result into a considerable costs reduction.

If SET is to be implemented, more patient-friendly IVF strategies might be considered. This will result into a lower number of redundant embryos, but more importantly, also in reduction of the risk of ovarian hyperstimulation syndrome. Minimal ovarian stimulation regimens or even natural cycle IVF can be applied as a low-risk, low-cost and patient-friendly procedure. Pelinck and colleagues reported an ongoing pregnancy rate of 7.2% in a systematic literature review of 1800 natural IVF cycles (27). Lukassen et al showed a live birth rate of 10.3% per cycle in 29 natural ICSI cycles in patients younger than 37 years of age (28). On the other hand, we have to consider that there will be no or a restricted selection of embryos available for transfer and it will result in a decrease of the cryoaugmentation effect. A cost-effectiveness study remains to be done.

The psychological and social impact of multiples on the host family must also not be underestimated. Mothers of IVF twins experience more parenting stress as compared with mothers of IVF singletons and natu-

rally conceived singletons (29;30). Parents of multiples are more likely to get divorced and mothers of twins suffer more often from fatigue and depression (31;32). In our study many couples refused to participate because they desired a twin over a singleton gestation. Ryan et al recently showed in a prospective analysis that 20% of women with infertility desired multiples, which was associated with nulliparity and lower family income (33). It is the question whether the patients in our study were aware of the practical, financial and emotional impact that the birth of twins can have on their lives. On the other hand, we did not study whether there was more emotional stress for the couples in case more than one cycle was needed to become pregnant of a singleton or to undergo several IVF cycles to have two singletons instead of one twin. Moreover, insurance policies may play an important role in the acceptation of single embryo transfer. At the time this study was performed the Dutch government reimbursed a maximum of three IVF cycles, nowadays the patient has to pay the first out of three IVF cycles. As a consequence, probably more patients will insist on double embryo transfer, which will cost the government relatively much more money.

We are aware of the rather small sample size of our study. Retrospectively, to show a significant difference of 10% between the live birth rate of SET (26%) and DET (36%), 90 patients per group would have been needed (α error = 0.10 and β error = 0.20). Therefore, it is necessary to keep monitoring the success rate thoroughly in the future after implementing SET in daily practice and through well designed randomized controlled trials and possible meta-analysis to get more precise estimates of the success rate.

In conclusion, two cycles with single embryo transfer are equally effective as one cycle with double embryo transfer, and the medical costs per live birth up to six weeks after delivery are about the same. This favours the SET strategy because of the dramatic difference in multiple pregnancy rates. When the lifetime costs for severe handicaps due to multiple pregnancies are included, almost 7,000 euro per live birth could be saved after implementing SET in this patient group. Therefore, the DET strategy should be abandoned completely in this high-risk group, in favour of the strategy with 2 cycles with SET. This will save the government not only a lot of money but most of all the patients and their offspring will be saved from a large range of medical and socio-emotional problems.

References

1. Coetsier T, Devroey P, Dhont M, Edwards RG, Evers H, Hagglund L et al. Prevention of twin pregnancies after IVF/ICSI by single embryo transfer. Hum.Reprod. 2001;16(4):790-800.
2. Crosignani PG, Rubin BL. Multiple gestation pregnancy. Hum.Reprod. 2000;15(8):1856-64.
3. Senat MV, Ancel PY, Bouvier-Colle MH, Breart G. How does multiple pregnancy affect maternal mortality and morbidity? Clin.Obstet.Gynecol. 1998;41(1):79-83.
4. Sebire NJ, Jolly M, Harris J, Nicolaides KH, Regan L. Risks of obstetric complications in multiple pregnancies: an analysis of more than 400 000 pregnancies in the UK. Prenat.Neonatal Med. 2001;6(2):89-94.
5. Scher AI, Petterson B, Blair E, Ellenberg JH, Grether JK, Haan E et al. The risk of mortality or cerebral palsy in twins: A collaborative population-based study. Pediatric Research 2002;52(5):671-81.
6. Martin, J. A., Hamilton, B. E., Ventura, S. J., Menacker, F, Park, M. M., and Sutton, P. D. Births: final data for 2001. Natl.Vital.Stat.Rep. 2002;51(2):1-102.
7. Luke B, Keith LG. The Contribution of Singletons, Twins and Triplets to Low-Birth-Weight, Infant-Mortality and Handicap in the United-States. J.Reprod.Med. 1992;37(8):661-6.
8. Callahan TL, Hall JE, Ettner SL, Christiansen CL, Greene MF, Crowley WF, Jr. The economic impact of multiple-gestation pregnancies and the contribution of assisted-reproduction techniques to their incidence. N.Engl.J.Med. 1994;331(4):244-9.
9. Wolner-Hanssen P, Rydhstroem H. Cost-effectiveness analysis of in-vitro fertilization: estimated costs per successful pregnancy after transfer of one or two embryos. Hum.Reprod. 1998;13(1):88-94.
10. De Sutter P, Gerris J, Dhont M. A health-economic decision-analytic model comparing double with single embryo transfer in IVF/ICSI. Hum.Reprod. 2002;17(11):2891-6.
11. Gerris J, De Sutter P, De Neubourg D, Van Royen E, Vander Elst J, Mangelschots K et al. A real-life prospective health economic study of elective single embryo transfer versus two-embryo transfer in first IVF/ICSI cycles. Hum.Reprod. 2004;19(4):917-23.
12. Strandell A, Bergh C, Lundin K. Selection of patients suitable for one-embryo transfer may reduce the rate of multiple births by half without impairment of overall birth rates. Hum.Reprod. 2000;15(12):2520-5.

13. Vilska S, Tiitinen A, Hyden Granskog C, Hovatta O. Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. Hum.Reprod. 1999;14(9):2392-5.
14. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de MM, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. Hum.Reprod. 1999;14(10):2581-7.
15. Martikainen H, Tiitinen A, Tomas C, Tapanainen J, Orava M, Tuomivaara L et al. One versus two embryo transfer after IVF and ICSI: a randomized study. Hum.Reprod. 2001;16(9):1900-3.
16. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: a prospective randomized trial. Fertil. Steril. 2004;81(3):551-5.
17. Thurin A, Hausken J, Hillensjo T, Jablonowska B, Pinborg A, Strandell A et al. Elective single-embryo transfer versus double-embryo transfer in in vitro fertilization. N.Engl.J.Med. 2004;351(23):2392-402.
18. Lukassen HGM, Schönbeck Y, Adang E, Braat DDM, Zielhuis G, Kremer JAM. Cost analysis of singleton versus twin pregnancies after in vitro fertilization. Fertil.Steril. 2004;81:1240-6.
19. Steer CV, Mills CL, Tan SL, Campbell S, Edwards RG. The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. Hum.Reprod. 1992;7(1):117-9.
20. Quinn P, Kerin JF, Warnes GM. Improved Pregnancy Rate in Human Invitro Fertilization with the Use of A Medium Based on the Composition of Human Tubal Fluid. Fertil.Steril. 1985;44(4):493-8.
21. Van Steirteghem A, Tournaye H, Van der Elst J, Verheyen G, Liebaers I, Devroey P. Intracytoplasmic sperm injection three years after the birth of the first ICSI child. Hum.Reprod. 1995;10(10):2527-8.
22. Testart J, Lassalle B, Belaischallart J, Hazout A, Forman R, Rainhorn JD et al. High Pregnancy Rate After Early Human-Embryo Freezing. Fertil. Steril. 1986;46(2):268-72.
23. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Vercruyssen M, Barudy-Vasquez J et al. Elective single day 3 embryo transfer halves the twinning rate without decrease in the ongoing pregnancy rate of an IVF/ICSI programme. Hum.Reprod. 2002;17(10):2626-31.
24. Gardner DK, Schoolcraft WB. In-vitro culture of human blastocysts. In: Jansen R, Mortimer D, editors. Towards Reproductive Certainty:

Fertility and Genetics Beyond 1999. Parthenon Press, Carnforth, UK; 1999. p. 378-88.
25. Papiernik E. Social Cost of Twin Births. Acta Genet.Med.Gemellol. 1983;32(2):105-11.
26. Tiitinen A, Halttunen M, Harkki P, Vuoristo P, Hyden-Granskog C. Elective single embryo transfer: the value of cryopreservation. Hum.Reprod. 2001;16(6):1140-4.
27. Pelinck MJ, Hoek A, Simons AHM, Heineman MJ. Efficacy of natural cycle IVF: a review of the literature. Hum.Reprod. Update 2002;8(2):129-39.
28. Lukassen HGM, Kremer JAM, Lindeman EJM, Braat DDM, Wetzels AMM. A pilot study of the efficacy of intracytoplasmic sperm injection in a natural cycle. Fertil.Steril. 2003;79(1):231-2.
29. Glazebrook C, Sheard C, Cox S, Oates M, Ndukwe G. Parenting stress in first-time mothers of twins and triplets conceived after in vitro fertilization. Fertil.Steril. 2004;81(3):505-11.
30. Pinborg A, Loft A, Schmidt L, Andersen AN. Morbidity in a Danish National cohort of 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons: health-related and social implications for the children and their families. Hum.Reprod. 2003;18(6):1234-43.
31. Hay DA, Gleeson C, Davies C, Lorden B, Mitchell D, Paton L. What Information Should the Multiple Birth Family Receive Before, During and After the Birth. Acta Genet.Med.Gemellol. 1990;39(2):259-69.
32. Thorpe K, Golding J, Macgillivray I, Greenwood R. Comparison of Prevalence of Depression in Mothers of Twins and Mothers of Singletons. BMJ 1991;302(6781):875-8.
33. Ryan GL, Zhang SH, Dokras A, Syrop CH, Van Voorhis BJ. The desire of infertile patients for multiple births. Fertil.Steril. 2004;81(3):500-4.

This study was supported by the VAZ (vereniging voor academische ziekenhuizen) and CVZ (college voor zorgverzekeringen).

4

A pilot study of the efficacy of intracytoplasmic sperm injection in a natural cycle

Marieke H.G.M. Lukassen
Jan A.M. Kremer
Emiel J.M. Lindeman
Didi D.M. Braat
Alex M.M. Wetzels

Department of Obstetrics and Gynecology,
University Medical Center Nijmegen,
Nijmegen, the Netherlands.

Fertility and Sterility 2003; 79:231-232

Introduction

The first successful pregnancy resulting from IVF occurred during an unstimulated normal menstrual cycle (1). This approach was soon abandoned in favor of controlled ovarian hyperstimulation leading to improved pregnancy rates. However, ovarian stimulation has many disadvantages, including patient burden; risk for various adverse conditions, such as the ovarian hyperstimulation syndrome, multiple pregnancies, and cancer in the long term; ethical dilemmeas, such as storage and disposal of extra embryos; and high costs (2).

These disadvantages have prompted many researchers to again investigate IVF in the natural cycle, especially because IVF technique and LH monitoring have been improved (2). It has been suggested that IVF in the natural cycle is best performed in patients with tubal factor infertility, because both male and female gametes should be normal (3). However, the efficacy of ICSI in the natural cycle among patients with severe oligozoospermia has now been investigated serially and systematically.

Materials and methods

We performed a pilot study to investigate the efficacy of ICSI in 29 natural cycles of 25 couples. The study was performed at the Department of Obstetrics and Gynecology of the University Medical Center Nijmegen, Nijmegen, the Netherlands. Inclusion criteria were female age <37 years, infertility due to severe male factor with indication for ICSI (total motile sperm count < 1×10^6), regular menstrual cycle of 26 to 32 days, a baseline FSH level <10 IU/L, and all couples participating in the study gave written informed consent.

Serial transvaginal ultrasonography was performed to monitor follicle growth and endometrial thickness from day 8 or 9, depending on the length of the cycle. At each visit, serum levels of estradiol and LH were measured. When the dominant follicle was at least 16 mm in diameter as measured in two directions and the morning serum LH level was 15 IU/L or less, ovulation was triggered with 10,000 IU of hCG (Pregnyl®; Organon, Oss, the Netherlands). Excluded were patients with abnormal follicular growth, defined as stagnation or decrease in diameter of the dominant follicle in three consecutive measurements.

Table 1. Results of 29 natural ICSI cycles.

	All cycles (%/cycle)	No. of cycles with elevated LH level (n)	No. of cycles without elevated LH levels (n)
No. of cycles started	29	10	19
No. of cycles cancelled	3 (10.3)	3	0
Premature ovulation	2 (6.9)	0	2
Oocyte retrieval	24 (82.8)	7	17
successful	17 (58.6)	6	11
not successful	7 (24.1)	1	6
Result of ICSI	14 (48.3)	6	8
Fertilization (2 pronuclei)	14 (48.3)	6	8
Triploidy (3 pronuclei, no ET)	1 (3.4)	1	0
No fertilization	0	0	0
Abnormal zygote, no ET	1 (3.4)	1	0
Result of ET	12 (41.4)	4	8
Pregnancy	3 10.3)	0	3
Live birth	3 (10.3)	0	3

Transvaginal oocyte retrieval was scheduled 34 hours after hCG administration. If the serum LH value was elevated (>15 IU/L), oocyte retrieval was performed the next morning. No local or general anaesthesia was given because of the supposed low burden of the quick ovum-pickup for the patient.

After retrieval, the oocytes were denuded before injection was performed. Freshly ejaculated semen were used for ICSI. The morning after injection, the oocytes were examined for the presence of 0, 1, 2 or ≥3 pronuclei. On day 3 after oocyte retrieval (64 to 72 hours after injection), embryos were scored for cell number and embryo quality. Embryo transfer was then performed. No luteal support was given since no pituitary desensitization with gonadotrophin-releasing hormone analogues was realized.

On day 15 after embryo transfer a pregnancy test was performed. Five weeks after embryo transfer, clinical pregnancy was confirmed by ultrasonographic evidence of an intrauterine gestational sac.

Results

Table 1 shows our findings. Twenty-five patients underwent 29 ICSI cycles (4 patients participated in 2 consecutive cycles). The median female age was 31 ± 3.1 years. The median duration of infertility was 2.1 ± 2.4 years. Three cycles were cancelled, 2 because of abnormal follicular development and 1 because of an unexpected irregular menstrual cycle of 36 days. Two patients ovulated between the hCG trigger and oocyte retrieval even though no increased LH value had been detected.

Oocyte retrieval was performed in a total of 24 cycles in which the mean follicle diameter was 17.2 ± 1.0 mm and the estradiol level was 790 ± 240 pg/L on the day of hCG administration. In 7 cycles, the LH value was increased on the morning of the last check (Table 1). The mean endometrial thickness was 8.9 ± 1.5 mm on the day of hCG injection.

Embryo transfer was performed in 12 patients (41.4% per cycle). Three patients became pregnant and delivered a healthy infant (25% per embryo transfer). Thus, the live birth rate per started cycle was 10.3% (3 of 29 cycles). Quality scores were grade 4 (no fragmentation) in three embryos, grade 3 (<10% fragmentation) in three embryos, and grade 2 (10% to 20% fragmentation) in two embryos. Three pregnancies resulted from two grade 4 and one resulted from grade 2 eight-cell embryos.

Discussion

As far as we know, this is the first study investigating the efficacy of ICSI only in the natural cycle in a series of patients. Although male factor infertility is associated with lower fertilization rates in natural-cycle IVF (4), we found a high fertilization rate in retrieved oocytes after ICSI. All injected oocytes became fertilized, and only one fertilized oocyte did not develop to a cleavage-stage embryo. Thus, no oocyte was damaged by the ICSI procedure.

A disadvantage of natural-cycle ICSI is the high cancellation rate because of inadequate follicular development or premature LH surges, despite selection of patients with a regular menstrual cycle and absence of a female factor of infertility. When cycles with a LH surge are taken into account (n = 10), the cancellation rate in our study was 44.8% (13 of 29 cycles) (Table 1).

In contrast to the findings of Lenton et al. (5), we found no premature ovulation or immature oocytes (absence of a polar body) in patients with an increased LH value. Human chorionic gonadotropin was administered in five of these cases and was not administered in two cases, according to the physician's judgment. The oocyte retrieval rate per pick-up was higher in the group with a LH surge than in the group without a LH surge (85.7% and 64.7%, respectively), although this diference was not statistically significant. However, no pregnancies were obtained in the six patients with a LH surge. From these data, we cannot draw the conclusion that it is better to cancel cycles in which a LH surge is measured.

An interesting observation was the large variety in embryo quality (cell number and fragmentation on day 3). It is possible that during the process of follicular growth, one of the best follicles with a relatively good quality oocyte is selected by nature to ovulate. A further improvement could be expected by the lack of a negative effect of hyperstimulation on embryo quality, as has been described in murine models (6). In contrast to these expectations, only 3 of 13 embryos in our study were of excellent quality on day 3 (> 7 cells and no fragmentation). Two of these embryos implanted and led to the birth of a healthy infant. The third implanted embryo had eight cells and was of grade 2 quality. Some patients had an embryo with moderate quality in the natural cycle but had excellent quality embryos in a subsequent hyperstimulated cycle, in which 9 of 18 patients became pregnant. Thus, if natural selection of the best oocyte occurs during follicular growth, it does not find expression in the quality of the embryos in our study.

In conclusion, our study shows that ICSI in the natural cycle may be a suitable alternative to ICSI in stimulated cycles. The ongoing pregnancy of 10.3% per started cycle is satisfactory considering the low burden, low costs and low risks. Moreover, this protocol can be performed on a monthly basis, because there is no need for the ovaries to recover from hormonal stimulation. Extrapolation of the 10.3% live birth rate after one natural cycle yields a calculated cumulative live birth rate after three natural cycles of 28%. We also found a rate of 32% after one stimulated ICSI treatment in women younger than 37 years in our setting in 2000 (unpublished data). Thus, in the same time span the pregnancy rates are similar. We believe that natural-cycle ICSI may be a suitable alternative to stimulated ICSI cycles.

References

1. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet 1978;2(8085):366.
2. Nargund G, Waterstone J, Bland J, Philips Z, Parsons J, Campbell S. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. Hum.Reprod. 2001;16(2):259-62.
3. Janssens RM, Lambalk CB, Vermeiden JP, Schats R, Schoemaker J. In-vitro fertilization in a spontaneous cycle: easy, cheap and realistic. Hum.Reprod. 2000;15(2):314-8.
4. Daya S, Gunby J, Hughes EG, Collins JA, Sagle MA, YoungLai EV. Natural cycles for in-vitro fertilization: cost-effectiveness analysis and factors influencing outcome. Hum.Reprod. 1995;10(7):1719-24.
5. Lenton EA, Cooke ID, Hooper M, King H, Kumar A, Monks N et al. In vitro fertilization in the natural cycle. Baillieres Clin.Obstet.Gynaecol. 1992;6(2):229-45.
6. Wetzels AM, Artz MT, Goverde HJ, Bastiaans BA, Hamilton CJ, Rolland R. Gonadotropin hyperstimulation influences the 35S-methionine metabolism of mouse preimplantation embryos. J.Assist.Reprod.Genet. 1995;12(10):744-6.

PART II

Immunological Aspects

5

The proportion of follicular fluid CD16^{+}CD56^{DIM} NK cells is increased in IVF-patients with idiopathic infertility

Marieke H.G.M. Lukassen[1]
Arnold van der Meer[2]
Marie-José C. van Lierop[3]
Emiel J.M. Lindeman[1]
Irma Joosten[2]
Didi D.M. Braat[1]

Departments of Obstetrics and Gynaecology[1] and Bloodtransfusion and Transplantation Immunology[2], University Medical Center Nijmegen, Nijmegen, the Netherlands.

Department of Pharmacology[3], NV Organon, Oss, the Netherlands.

Journal of Reproductive Immunology 2003; 60:71-84

Abstract

One fifth of all IVF-patients suffer from idiopathic infertility. A low fertilization rate is one of the most characteristic features of IVF in this group, probably caused by oocyte dysfunction. We speculate that an altered lymphocyte profile in follicular fluid may affect oocyte function and thus play a role in idiopathic infertility. Therefore, we compared levels of lymphocyte populations present in follicular fluid (FF) of eleven patients with idiopathic infertility (study group) with 29 patients in the control group i.e. severe male factor infertility (n=17) or tubal factor infertility (n=12). Triple color flow cytometry was used to discriminate between T cells and NK cell subpopulations. In the idiopathic infertility group a shift from T cells to NK cells was observed in FF as compared to the control group, mainly caused by a significant higher level of NK cells, 20.3% and 13.6% ($p<0.05$), respectively. This high level of NK cells was due to a rise of the $CD16^{+}CD56^{dim}$ NK cell subset. In peripheral blood the NK cell levels showed a similar, although not significant trend ($p=0.08$). As the $CD16^{+}CD56^{dim}$ NK cell subpopulation is known for its cytotoxic properties, this subpopulation may negatively affect folliculogenesis and oocyte maturation, which is reflected by a diminished fertilization rate in the idiopathic infertility group. An altered lymphocyte profile in follicular fluid could therefore influence fertility in these patients.

Introduction

In about one fifth of the patients who start In-vitro Fertilization (IVF) the cause of their fertility problem is unknown (1). Patients with unexplained infertility form a heterogeneous population whose etiology remains elusive, despite profound infertility investigations of both partners. It is known that the fertilization rate of oocytes from patients with unexplained infertility is lower as compared to oocytes from patients suffering from tubal factor infertility, resulting more frequently in total fertilization failure in the first group (2-4). Fertilization failure can be caused by unsuspected sperm dysfunction and/or oocyte dysfunction (5;6).

In the ovary immunological factors play an important role in folliculogenesis and corpus luteum development (7;8). Follicular fluid (FF) provides the environment in which oocyte maturation occurs. Follicular fluid may therefore influence the quality of the oocyte, and thus be linked to fertilization and early embryonic development. Until now in follicular fluid the presence and function of many cytokines have been determined (9-14). However, there is only limited information from dated studies evaluating the immune cells present in FF responsible for the production of those cytokines (15-20). These studies have demonstrated the presence of the following leukocyte populations in FF of women undergoing IVF treatment: monocytes-macrophages, B cells, T cells and natural killer (NK) cells.

It is known that NK cells play an important role in human reproduction. Two phenotypically and functionally distinct NK cell subsets can be distinguished by CD56 surface density expression. The $CD16^{+}CD56^{dim}$ NK cells have cytotoxic properties and $CD16^{-}CD56^{bright}$ NK cells produce mainly immunoregulatory cytokines (21). The NK cell population in the uterus predominantly comprises of $CD16^{-}CD56^{bright}$ NK cells, which are supposed to be crucial at the time of implantation by controlling trophoblast invasion (22;23).

The main population (90%) of peripheral blood NK cells is $CD16^{+}CD56^{dim}$. Implantation failure in IVF and idiopathic infertility in IVF patients was associated with a high level of $CD16^{+}CD56^{dim}$ NK cells (24;25).

We hypothesize that a disturbance in the local cellular immune system of the follicle might cause aberrant oocyte development which can

reveal a possible explanation for the 'idiopathic' cause of infertility in this group of patients. We determined the levels of T and NK cell subsets by flowcytometric analysis in separately collected follicular fluids of patients with idiopathic infertility and compared these levels to FF of a control group consisting of couples with severe male and tubal factor infertility.

Materials and methods

Patients

After obtaining an informed consent according to the Medical Ethical Review Committee of the University Medical Center Nijmegen, 63 follicular fluid samples were collected from 40 women undergoing IVF. From these 40 patients, 11 patients were categorized in the study group (idiopathic infertility) and 29 patients in the control group i.e. male factor infertility (n=17) or tubal factor infertility (n=12).

Couples were diagnosed as having idiopathic infertility if no abnormality was found during the full infertility investigation and they had been suffering from idiopathic subfertility for at least 3 years, in spite of several intrauterine inseminations. Severe male factor infertility with indication for intracytoplasmic sperm injection (ICSI) was diagnosed if at least two semen samples yielded less than 0.5 million progressively motile spermatozoa after processing by 80% Puresperm® (Nidacon International, Götenborg, Sweden) gradient centrifugation. Tubal factor infertility was defined as bilateral or unilateral tubal occlusion in combination with infertility for at least two years. In these patients no hydrosalpinges were diagnosed by ultrasound.

IVF-procedure

Pituitary down-regulation (long protocol) was achieved using a GNRH analogue (Decapeptyl®; Ferring, the Netherlands). Multiple follicle growth was achieved using recombinant FSH (Puregon®; Organon, Oss, the Netherlands). Thirty-six hours after hCG injection (Pregnyl®; Organon, Oss, the Netherlands) we performed transvaginal oocyte retrieval.

For this study the fluids of the two largest follicles in each ovary were collected and assessed as follows: the fluid of the first follicle was collected in a tube. The needle was removed and flushed with Phosphate

Buffered Saline (PBS) plus heparine and penicilline, to maximize oocyte retrieval. This flush medium was collected into another tube in order to prevent contamination with the previous punctured follicle. Then the needle was re-injected to puncture the second largest follicle. This fluid was collected separately as well. The same procedure took place in the other ovary. Because of the patient's burden of multiple vaginal wall punctures it was not considered ethical to collect more than four FF's separately per patient.

Leukocyte isolation

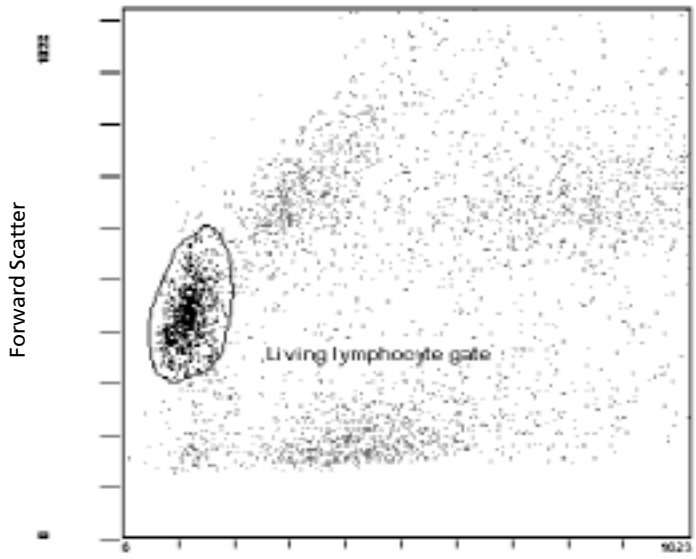
Follicular fluid was centrifuged at 1400 rpm for 5 minutes. After discarding the supernatant the remaining cells were washed in RPMI-1640 supplemented with 10% human serum, sodium pyruvate and 1% penicillin-streptomycin (GIBCO, Grand Island, NY). After centrifugation, the cells were resuspended in 2 ml lysis buffer (NH_4Cl, K-EDTA) to remove erythrocytes and incubated on ice for 5 minutes. The cells were washed again after adding 13 ml of Phosphate Buffered Saline (PBS). Cells were resuspended in fluorescence activated cell sorting (FACS) buffer (PBS containing 0.5% bovine serum albumin (BSA)) and the leukocytes were counted. Leukocytes were present at a mean concentration of $90x10^3$ cells/ml in FF. There was no significant difference in the numbers of leukocytes isolated from FF of the patient group (mean ± SD; $0.42x10^6$ ± $0.41\ x10^6$ leukocytes) and the control group ($0.25x10^6$ ± $0.21\ x10^6$ leukocytes).

During oocyte retrieval also a peripheral blood sample of each patient was taken. Peripheral blood mononuclear cells (PBMC) were isolated from these samples by Ficoll-Hypaque density centrifugation (Lymphoprep, Nycomed, Oslo, Norway). The leukocyte fraction was washed twice in PBS and counted.

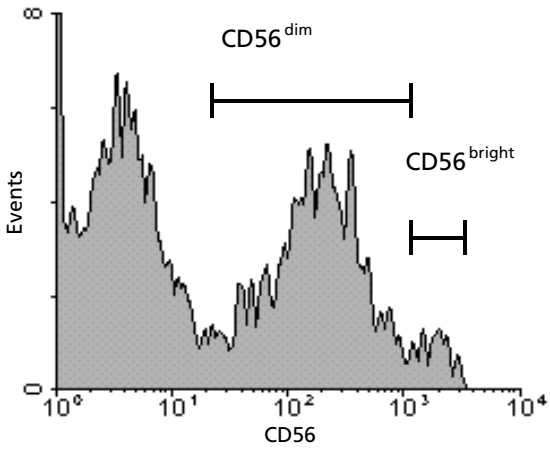
Flow cytometric labeling and analysis

Cells were phenotypically analyzed by a one-step triple labeling procedure. $5x10^4$ cells were stained directly using a combination of CD3-FITC/CD45-PE (Dako, Glostrup, Denmark)/CD56-Cy5 (Coulter Immunotech, Marseille, France), and CD16-FITC (Dako, Glostrup, Denmark)/CD3-PE/CD56-Cy5.

The follicular lymphocytes were analyzed blinded with regard to the origin of the samples. Analysis was as follows: a gate was set around the

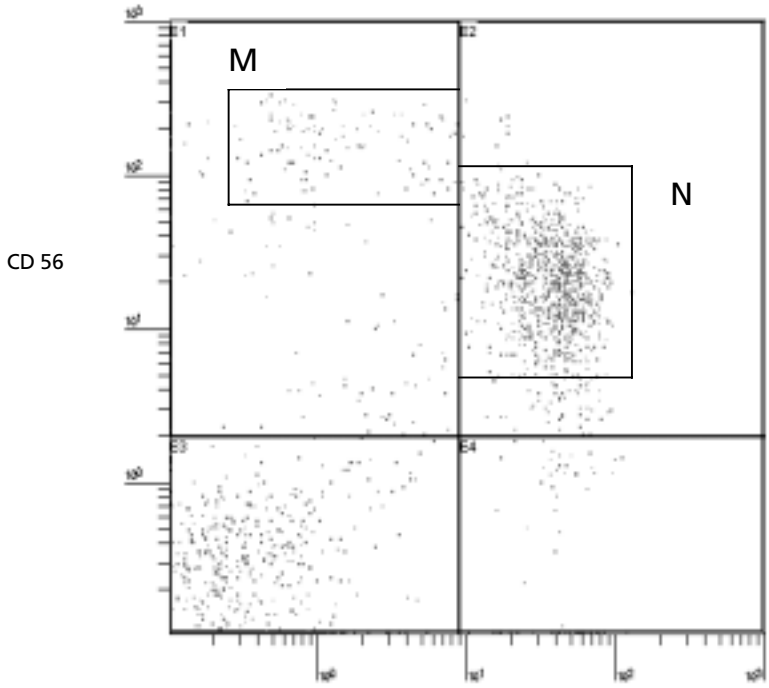


(A)



(B)

Figure 1. (A) Dot plot showing forward and site scatter of mononuclear cells present in FF. A gate was set around the living lymphocytes for analysis of the T, NK and NK-T cells.
(B) Fluorescence histogram of the CD56 expression level ($CD56^{dim}$, $CD56^{bright}$) of $CD45^{+}$ leukocytes in FF of a patient with male factor infertility determined by flow cytometry.
(C) FACS plot showing $CD45^{+}$ leukocytes double-stained for CD16 and CD56. A gate was set around the $CD3^{-}$ population to exclude NK-T cells. The ($CD3^{-}$)$CD16^{+}CD56^{dim}$ (*M*) and ($CD3^{-}$) $CD16^{-}CD56^{bright}$ NK cell (*N*) subpopulations are indicated. Data from one representative FF of the same patient as in Fig. 1A are shown.



live lymphocyte population, as indicated by Propidium Iodide (PI, 5 mg/ml) staining, in a forward and side scatter dot plot (Fig. 1A). Backgating on CD3 and CD56 confirmed that practically all lymphocytes were located in the gate. The percentage of NK, NKT and T cells were measured, as well as the intensity of fluorescence.

The CD45 marker was used to identify the total leukocyte population in the gate, T cells were defined as $CD3^+$ cells, NK cells as $CD56^+CD3^-$ cells and NKT cells as $CD3^+CD56^+$ cells. Two distinct subsets of NK cells were identified by cell surface density of CD56 and the presence of CD16 antigen, i.e. $CD16^-CD56^{bright}$ and $CD16^+CD56^{dim}$ (Fig. 1B and C). Isotype matched antibodies were used to define marker settings (Dako, Glostrup, Denmark and Immunotech, Marseille, France).

The samples were run on a Coulter Epics XL Flowcytometer (Beckman Coulter, Fullerton, CA, USA). Analysis of the data was performed using Coulter Epics Expo 32 software (Beckman Coulter, Fullerton, CA, USA).

Statistical methods

NK, NKT and T cell numbers were expressed as a percentage of the number of $CD45^+$ cells. Multiple follicles were sampled from a single patient. The variation in lymphocyte levels obtained from a single patient was significantly smaller than the variation observed between patients, allowing us to average lymphocyte levels of each follicle obtained from one patient. The *t*-test for independent samples was used, after a log-transformation if appropriate, to test for statistical significant differences in mean values between the idiopathic infertility group and the control group. Paired student's *t*-test was used to compare means between FF and peripheral blood within patients. The dichotomized data were analyzed using the χ^2-test. P-values ≤ 0.05 were considered significant. All analyses were performed two-sided. Statistics Package for Social Sciences was used.

Results

Distinct lymphocyte levels are observed in FF versus peripheral blood

Significant differences in lymphocyte levels were found between follicular fluid and peripheral blood per patient in the control group. The percentage of T cells was lower in FF than in peripheral blood, while the percentages of $CD16^-CD56^{bright}$ NK cells, NKT cells and the remaining mononuclear cells, i.e. particularly B-lymphocytes and monocytes ($CD45^+CD3^-CD56^-$) were significantly higher in FF as compared to peripheral blood (Table 1). Thus, we show that the cellular composition of peripheral blood and follicular fluid is different, despite possible contamination of FF with ovarian blood lymphocytes.

A high FF level of $CD16^+CD56_{dim}$ NK cells is observed in idiopathic infertility

From the 40 patients, with a median number of 2 FF per patient, 11 patients were categorized in the study group (n=22 FF) and 29 in the control group (n=41 FF). There was no significant difference (p-value < 0.05) between the study group and the control group with respect to age, the level of FSH on cycle day 3 and history of previous conception (Table 2).

In both groups an equal dosage of GnRH analogue and hCG was used and also the duration of the treatment was equal. The total amount

Table 1. Levels of lymphocyte populations in FF and peripheral blood

Cells	FF		Peripheral blood	
	Idiopathic (n = 11)	**Control** (n = 29)	**Idiopathic** (n = 11)	**Control** (n = 29)
NK cells ($CD45^+CD3^-CD56^+$)	20.3 (7.8)[a]	13.6 (8.1)	18.3 (8.2)[b]	13.2 (6.4)
$CD16^+CD56^{dim}$NK[c]	18.9 (7.4)[a]	13.0 (7.8)	17.0 (8.0)	11.8 (6.2)
$CD16^-CD56^{bright}$NK[c]	1.5 (0.8)	1.9 (1.8)	1.3 (0.8)	1.3 (0.8)[d]
T cells ($CD45^+CD3^+CD56^-$)	67.2 (10.5)	71.6 (10.5)	72.4 (10.2)	76.0 (8.5)[d]
NKT cells ($CD45^+CD3^+CD56^+$)	3.8 (2.4)	4.1 (4.1)	2.7 (1.6)	3.2 (3.1)[d]
B-cells, Monocytes etc. ($CD45^+CD3^-CD56^-$)	8.8 (4.3)	10.7 (4.4)	6.6 (3.6)	7.7 (4.8)[d]

Values represent mean (SD) percentage of lymphocytes.

[a] Significant difference ($p<0.05$) in FF between idiopathic infertility and control group.

[b] Significant difference ($p<0.05$) in peripheral blood between idiopathic infertility and control group.

[c] In the control group the levels of the NK cell subsets in FF and PBMC are determined in only 24 patients.

[d] Significant difference ($p<0.05$) in control group between FF and peripheral blood.

recombinant FSH used for multiple follicle growth was 2,590 IU in the study group and 2,435 IU in the control group. This was not significantly different.

In the study group 68 % of the oocytes fertilized successfully, while one patient experienced total fertilization failure. In the control group successful fertilization took place of 63% of the oocytes. The number of patients experiencing total fertilization failure in this group was two.

FCM analysis of follicular fluid revealed a shift from T cells to NK cells in the idiopathic infertility group as compared to the control group, exemplified by a significantly higher level of NK cells, 20.3% and 13.6%, respectively ($p<0.05$, Table 1, Fig. 2). This increase of NK cells was due to a significant relative rise of the $CD16^+CD56^{dim}$ NK cell, but not the $CD16^-CD56^{bright}$ NK cell subset. The level of $CD3^+CD56^+$ NKT cells and remaining mononuclear cells, like monocytes and B-lymphocytes, in FF from the study group was not different from the control group.

Table 2 Patient characteristics

	Idiopathic infertility	Control group
Number of patients (*n*)	11	29
Age[a] (year)	34.6 ± 4.1	34.0 ± 4.5
FSH[a,b] (IU/L)	6.8 ± 1.4	7.1 ± 2.5
Primary infertility (%)	58	50

[a] Values represent mean ± SD.
[b] on cycle day 3.

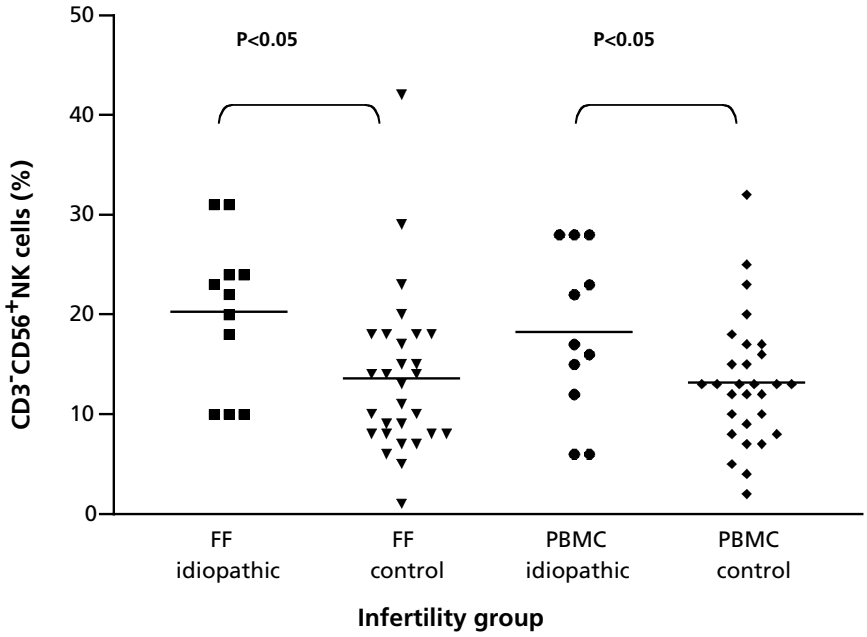


Figure 2. Graphical representation of the relationships between cause of infertility and lymphocyte levels in FF and peripheral blood (PBMC). The individually determined NK cell ($CD3^-CD56^+$) level was expressed as percentage of $CD45^+$ cells in patients with idiopathic infertility (*n*=11) and in a control group (*n*=29). The horizontal line shows the mean value. Braces indicate significant differences in levels of lymphocyte populations between the idiopathic and control group and/or between FF and PBMC.

Also, in peripheral blood of women suffering from idiopathic infertility there was a significantly higher level of NK cells observed as compared to the control group, 18.3% and 13.2%, respectively ($p<0.05$, Table 1, Fig. 2). A trend was observed towards an increase of the CD16^{+}CD56^{dim} NK cell subset ($p=0.08$). No difference was observed between the idiopathic infertility and control group for the NKT cell level and the level of the remaining mononuclear cells (Table 1).

Discussion

Follicular fluid provides the most suitable microenvironment for the oocyte and is very likely a key factor in determining growth and development of this particular oocyte. Therefore, a disturbance in the cellular immunity of follicular fluid might affect the oocyte. Our data show that in follicular fluid from patients suffering from idiopathic infertility a significant higher percentage of NK cells was present as compared to follicular fluids from a control group. This higher level of NK cells was exemplified by a relative increase of the CD16^{+}CD56^{dim} NK cell subpopulation. The excess of CD16^{+}CD56^{dim} NK cells in follicular fluid could reveal a cause for oocyte 'dysfunction' and therefore for the idiopathic infertility in this group.

It is believed that the NK cell subpopulations, CD16^{+}CD56^{dim} and CD16^{-}CD56^{bright}, represent functionally and phenotypically distinct subsets of NK cells with unique immunoregulatory roles in vivo. CD16^{+}CD56^{dim} NK cells have cytotoxic and antibody-dependent cellular cytotoxic properties, while CD16^{-}CD56^{bright} NK cells mainly produce immunoregulatory cytokines (21). The CD16^{-}CD56^{bright} NK cell population appears in relatively high number at the fetal-maternal interface of the decidua in the uterus and is strongly associated with successful implantation and placental maturation (23). The CD16^{+}CD56^{dim} NK cell subpopulation is negatively associated with reproductive events. Fukui *et al.* (24) reported higher cytotoxicity and CD16^{+}CD56^{dim} NK cell count in endometrial tissue of IVF patients experiencing an abortion compared to an ongoing pregnancy. As it is clear that NK cells play an important role in the uterus, it can be expected that NK cells are also playing a role in the ovary, i.e. follicle, influencing oocyte quality. Next to the study described in this paper, only one other study investigated the correlation

between cause of infertility and NK cell levels in follicular fluid (19). In contrast to our results, they found no correlation.

In peripheral blood a high proportion of $CD16^{+}CD56^{dim}$ NK cell population is negatively associated with reproduction, such as implantation failure during IVF, multiple IVF failures in idiopathic infertile women and recurrent spontaneous abortion (26-28). In line with the results of these reports, we found in peripheral blood of patients with idiopathic infertility a significant higher percentage of NK cells. Again, the level of $CD16^{+}CD56^{dim}$ NK cells (17.0%) in this group was higher, albeit not significantly, as compared to the control group (11.8%, P=0.08). So, also on systemic level we observed an altered lymphocyte profile in idiopathic infertility, but these differences were less outspoken. A generalized alteration in the cellular immunity of patients with idiopathic infertility could also lead to poor fertilization rates or perhaps poor implantation rates. We found no significant difference in the absolute number of leukocytes in follicular fluid between the patient group and the control group (mean ± SD is $0.42x10^6$ ± $0.41 x10^6$ and $0.25x10^6$ ± $0.21 x10^6$ leukocytes, respectively) when counted by light microscopy using a counting chamber. This suggests that there is not just a relative increase in NK cells but also an increase in the absolute number of $CD16^{+}CD56^{+}$ NK cells in the follicles of idiopathic patients. Yovel et al. (29), have shown that during the periovulatory phase of the menstrual cycle there is an increase in the number of NK cells in peripheral blood. Further studies using quantitative flowcytometric analysis with beads for standardization (as used by Yovel et al.(29)) will have to confirm the increase in the absolute number of NK cells in the follicles of patients suffering from idiopathic infertility.

We observed a quantitative difference in the levels of various lymphocyte populations between FF and peripheral blood, although the possibility that a part of the lymphocytes we found in FF are derived from blood of the local circulation of that particular follicle can not be excluded. Piccinni and co-workers (30) reported also a qualitative, tissue-specific difference in functionality between lymphocytes present in the cumulus cells of a follicle, producing a different cytokine amount and profile, as compared to peripheral blood lymphocytes. The observed differences raise the question whether the lymphocytes present in follicular fluid originate from a distinct population or are recruited from the peripheral blood circulation. It might be that these peripheral lymphocytes home to the follicle where they may undergo tissue-specific differ-

entiation. It is known that NK cells express a variety of adhesion molecules and chemokine receptors such as CCR2 and CCR5 (31) while follicular fluid is known to contain different chemoattracants such as IL-8 and MCP1 (32;33). In addition cytokines present in follicular fluid may play a role in activation or differentiation of lymphocyte subsets. It has for example been shown that the presence of IL-12 in follicular fluid, a cytokine that is capable of activating NK cells, is associated with a negative outcome in IVF treatment (32).

A low fertilization rate and subsequent high number of total fertilization failures is one of the most specific features of IVF treatment of patients with unexplained infertility (3;34) and is a manifestation of abnormal gamete interaction. In our clinic the frequency of total fertilization failure in patients with idiopathic infertility was 16% compared to 5.5% in the control group ($P<0.0001$). Similar data have been reported by others (3;4;35;36). Oocyte dysfunction was suggested to account for total fertilization failure in the idiopathic infertility group by Gabrielsen et al. (37). Hull and colleagues (6) demonstrated, by using donor semen, that in the unexplained infertility group both subtle sperm dysfunction as well as impaired function of the oocyte is responsible for the poor fertilization rate in this patient group. Concerning our study, it can be speculated that the altered lymphocyte profile, i.e. a high level of ($CD16^{+}CD56^{dim}$) NK cells, observed in follicular fluid of patients suffering from idiopathic infertility could be responsible for impaired oocyte maturation and fertilization. However, the functional role of these cells was not determined, because adequate numbers of cells could not be recovered.

In conclusion, the proportion of $CD16^{+}CD56^{dim}$ NK cells in follicular fluid of women suffering from idiopathic infertility is significantly higher than in controls. Whether idiopathic infertility is itself a cause or consequence of the altered immune cell composition of follicular fluid remains unclear and needs to be explored.

References

1. Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA et al. Population study of causes, treatment, and outcome of infertility. BMJ (Clin.Res.Ed) 1985;291(6510):1693-7.
2. Mahadevan MM, Trounson AO, Leeton JF. The relationship of tubal blockage, infertility of unknown cause, suspected male infertility, and endometriosis to success of in vitro fertilization and embryo transfer. Fertil.Steril. 1983;40(6):755-62.
3. Audibert F, Hedon B, Arnal F, Humeau C, Badoc E, Virenque V et al. Results of IVF attempts in patients with unexplained infertility. Hum.Reprod. 1989;4(7):766-71.
4. Lipitz S, Rabinovici J, Ben Shlomo I, Bider D, Ben Rafael Z, Mashiach S et al. Complete failure of fertilization in couples with unexplained infertility: implications for subsequent in vitro fertilization cycles. Fertil.Steril. 1993;59(2):348-52.
5. Mackenna A, Barratt CL, Kessopoulou E, Cooke I. The contribution of a hidden male factor to unexplained infertility. Fertil.Steril. 1993;59(2):405-11.
6. Hull MG, Williams JA, Ray B, McLaughlin EA, Akande VA, Ford WC. The contribution of subtle oocyte or sperm dysfunction affecting fertilization in endometriosis-associated or unexplained infertility: a controlled comparison with tubal infertility and use of donor spermatozoa. Hum.Reprod. 1998;13(7):1825-30.
7. Brannstrom M, Norman RJ. Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. Hum.Reprod. 1993;8(10):1762-75.
8. Brannstrom M, Pascoe V, Norman RJ, McClure N. Localization of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. Fertil.Steril. 1994;61(3):488-95.
9. Punnonen J, Heinonen PK, Teisala K, Kujansuu E, Jansen CT, Punnonen R. Demonstration of tumor necrosis factor-alpha in preovulatory follicular fluid: its association with serum 17 beta-estradiol and progesterone. Gynecol.Obstet.Invest 1992;33(2):80-4.
10. Wang LJ, Norman RJ. Concentrations of immunoreactive interleukin-1 and interleukin-2 in human preovulatory follicular fluid. Hum.Reprod. 1992;7(2):147-50.
11. Lopez BA, Newman GE, Phizackerley PJ, Laird E, Ross C, Barlow DH.

Platelet-activating factor levels in human follicular and amniotic fluids. Eur.J.Obstet.Gynecol.Reprod.Biol. 1992;46(1):39-44.
12. Cataldo NA, Giudice LC. Insulin-like growth factor binding protein profiles in human ovarian follicular fluid correlate with follicular functional status. J.Clin.Endocrinol.Metab 1992;74(4):821-9.
13. Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum.Reprod. 1997;12(5):1047-55.
14. Loret dM, Jr., Flores JP, Baumgardner GP, Goldfarb JM, Gindlesperger V, Friedlander MA. Elevated interleukin-6 levels in the ovarian hyperstimulation syndrome: ovarian immunohistochemical localization of interleukin-6 signal. Obstet.Gynecol. 1996;87(4):581-7.
15. Hill JA, Barbieri RL, Anderson DJ. Detection of T8 (suppressor/cytotoxic) lymphocytes in human ovarian follicular fluid. Fertil.Steril. 1987;47(1):114-7.
16. Droesch K, Fulgham DL, Liu HC, Rosenwaks Z, Alexander NJ. Distribution of T cell subsets in follicular fluid. Fertil.Steril. 1988;50(4):618-21.
17. Loukides JA, Loy RA, Edwards R, Honig J, Visintin I, Polan ML. Human follicular fluids contain tissue macrophages. J.Clin.Endocrinol.Metab 1990;71(5):1363-7.
18. Castilla JA, Sampalo A, Molina R, Samaniego F, Mozas J, Vergara F et al. Mononuclear cell subpopulations in human follicular fluid from stimulated cycles. Am.J.Reprod.Immunol. 1990;22(3-4):127-9.
19. Lachapelle MH, Hemmings R, Roy DC, Falcone T, Miron P. Flow cytometric evaluation of leukocyte subpopulations in the follicular fluids of infertile patients. Fertil.Steril. 1996;65(6):1135-40.
20. Castilla JA, Sampalo A, Gil T, Vergara F, Jr., Molina R, Herruzo AJ. CD8+ lymphocyte subsets in human follicular fluid. Fertil.Steril. 1992;57(5):1124-5.
21. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 2001;97(10):3146-51.
22. King A, Burrows T, Verma S, Hiby S, Loke YW. Human uterine lymphocytes. Hum.Reprod.Update. 1998;4(5):480-5.
23. King A. Uterine leukocytes and decidualization. Hum.Reprod.Update. 2000;6(1):28-36.
24. Fukui A, Fujii S, Yamaguchi E, Kimura H, Sato S, Saito Y. Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization. Am.J.Reprod.Immunol. 1999;41(6):413-22.

25. Kwak JY, Kwak FM, Ainbinder SW, Ruiz AM, Beer AE. Elevated peripheral blood natural killer cells are effectively downregulated by immunoglobulin G infusion in women with recurrent spontaneous abortions. Am.J.Reprod.Immunol. 1996;35(4):363-9.
26. Beer AE, Kwak JY, Ruiz JE. Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles. Am.J.Reprod.Immunol. 1996;35(4):376-82.
27. Ntrivalas EI, Kwak-Kim JY, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD et al. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. Hum.Reprod. 2001;16(5):855-61.
28. Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veerhoek M, Joosten I. Peripheral Natural Killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. Hum.Reprod. 2000;15(5):1163-9.
29. Yovel G, Shakhar K, Ben Eliyahu S. The effects of sex, menstrual cycle, and oral contraceptives on the number and activity of natural killer cells. Gynecol.Oncol. 2001;81(2):254-62.
30. Piccinni MP, Scaletti C, Mavilia C, Lazzeri E, Romagnani P, Natali I et al. Production of IL-4 and leukemia inhibitory factor by T cells of the cumulus oophorus: a favorable microenvironment for pre-implantation embryo development. Eur.J.Immunol. 2001;31(8):2431-7.
31. Nieto M, Navarro F, Perez-Villar JJ, del Pozo MA, Gonzalez-Amaro R, Mellado M et al. Roles of chemokines and receptor polarization in NK-target cell interactions. J.Immunol. 1998;161(7):3330-9.
32. Gazvani MR, Bates M, Vince G, Christmas S, Lewis-Jones DI, Kingsland C. Follicular fluid concentrations of interleukin-12 and interleukin-8 in IVF cycles. Fertil.Steril. 2000;74(5):953-8.
33. Kawano Y, Kawasaki F, Nakamura S, Matsui N, Narahara H, Miyakawa I. The production and clinical evaluation of macrophage colony-stimulating factor and macrophage chemoattractant protein-1 in human follicular fluids. Am.J.Reprod.Immunol. 2001;45(1):1-5.
34. Audibert, F., Hedon, B, Humeau, C., Arnal, F., and Viala, J. L. Responsibility of endometriosis in infertility:IVF results. 44th Annual Meeting of The American Fertility Society, October 10-13, Atlanta Abstract 055, s19. 1-1-1988.

35. Mackenna AI, Zegers-Hochschild F, Fernandez EO, Fabres CV, Huidobro CA, Prado JA et al. Fertilization rate in couples with unexplained infertility. Hum.Reprod. 1992;7(2):223-6.
36. Ruiz A, Remohi J, Minguez Y, Guanes PP, Simon C, Pellicer A. The role of in vitro fertilization and intracytoplasmic sperm injection in couples with unexplained infertility after failed intrauterine insemination. Fertil.Steril. 1997;68(1):171-3.
37. Gabrielsen A, Petersen K, Mikkelsen AL, Lindenberg S. Intracytoplasmic sperm injection does not overcome an oocyte defect in previous fertilization failure with conventional in-vitro fertilization and normal spermatozoa. Hum.Reprod. 1996;11(9):1963-5.

This study was in part supported by Organon NV, the Netherlands.

6

Hormonal stimulation for IVF treatment positively affects the CD56^{bright}/CD56^{dim} NK cell ratio of the endometrium during the window of implantation

Marieke H.G.M. Lukassen[1]
Irma Joosten[2]
Bram van Cranenbroek[2]
Marie-José C. van Lierop[3]
Hans Bulten[4]
Didi D.M. Braat[1]
Arnold van der Meer[2]

Departments of Obstetrics and Gynaecology[1], Bloodtransfusion and Transplantation Immunology[2] and Pathology[4], University Medical Center Nijmegen, Nijmegen, the Netherlands.

Department of Pharmacology[3], NV Organon, Oss, the Netherlands.

Molecular Human Reproduction 2004; 10:513-520

Abstract

The effects of hormone stimulation for IVF treatment on endometrial receptivity remain controversial. Since $CD56^{bright}$ NK cells in the endometrium positively contribute to implantation and decidualization, while $CD56^{dim}$ NK cells are negatively associated with reproduction, shifts in the balance between those cells will affect receptivity. Therefore, we compared the leukocyte composition in the endometrium of IVF women (n=20) with non-pregnant women (n=18) in a natural cycle, as a parameter for endometrial quality. Biopsies were obtained seven days after ovulation. Histological dating of the endometrium showed no increased endometrial advancement after IVF treatment as compared to the control group. Flow cytometric analysis of leukocyte subsets showed that hormonal stimulation positively affected the $CD56^{bright}$/ $CD56^{dim}$ ratio in the endometrium by a relative decrease in the cytotoxic $CD56^{dim}CD16^{+}$ NK cell numbers. The relative number of T cells remained unaffected, while the number of non-T and non-NK cells (i.e B-cells and macrophages) was higher in the IVF group. These effects were restricted to the endometrium and not observed in peripheral blood. Within the $CD56^{bright}$ population we could identify a distinct subset of NK cells ($CD56^{superbright}$) that was unique for the endometrium. We conclude that hormonal stimulation for IVF treatment positively affects the $CD56^{bright}$/ $CD56^{dim}$ ratio of the endometrium during the window of implantation and does not affect endometrial advancement.

Introduction

Ovarian stimulation for IVF treatment results in supraphysiological concentrations of sex steroids during the follicular and luteal phase of the menstrual cycle. The effect on endometrial receptivity remains controversial. Both positive and negative effects on pregnancy rates after IVF have been reported (1-4).

To date there are limited means to define endometrium quality. One parameter is the histological maturation of the endometrium in relation to the menstrual date. It has been stated that extreme endometrial advancement (> 2 days) results in lower pregnancy rates (5-7). Of interest with regard to IVF is that high levels of progesterone were found to be associated with advanced endometrial histology (8-10), dated by criteria of Noyes et al. (11).

Based on recent insights in NK cell function, the leukocyte composition in the endometrium could well be considered an alternative parameter for endometrial quality. During the menstrual cycle uterine NK cells increase dramatically in number after ovulation and reach a peak in the late secretory phase. In pregnancy their numbers remain high during early gestation, around 70% of the stromal leukocytes, and they come into close contact with invading extravillous trophoblast cells (5;10;12). The localization and the large numbers of NK cells found in the decidua during pregnancy suggest an important role in the decidualization (13).

The uterine NK cells (uNK) that are imputed to play an important role in implantation have a phenotype that differs from most peripheral blood NK cells. These so called $CD56^{bright}$ NK cells express the NK cell marker CD56 at high levels and lack the marker CD16. These NK cells have low cytotoxic activity but are potent producers of a variety of immunoregulatory cytokines and angiogenic growth factors contributing to endometrial angiogenesis (14-16). In contrast, most peripheral blood NK cells, but also a small part of the uterine NK cell fraction, is of the $CD56^{dim}CD16^{+}$ phenotype with high cytolytic activity and low cytokine production(15). This cytotoxic $CD56^{dim}$ NK cell population is negatively associated with pregnancy outcome (17-20).

Activation of NK cells is tightly regulated by a set of activating and inhibitory receptors on the cell surface. These receptors interact with HLA molecules expressed on the invading trophoblast (such as HLA-C, HLA-E and HLA-G) and thereby affect cytokine production and cytoly-

tic activity of maternal uNK cells (21-24). It is believed that each individual NK cell and the NK cell subsets expresses a unique combination of natural killer cell receptors depending on their functional role.

During the menstrual cycle as well as throughout pregnancy the leukocyte numbers vary, therefore an interaction with sex steroids is suggested, although it is not clear yet whether this is a direct or an indirect mechanism. DeLoia et al. have shown that the total number of lymphocytes in endometrium as well as in peripheral blood, particularly the NK cells, increases under influence of supraphysiological estrogen levels (25).

To determine the influence of ovarian stimulation on shifts in endometrial leukocyte populations, and in particular the NK cell subsets, we compared leukocyte populations in the endometrium of IVF patients with naturally cycling women during the window of implantation. By analyzing shifts in the beneficial $CD56^{bright}$ NK cells and the harmful $CD56^{dim}$ NK cells, we want to answer the question whether hormonal stimulation during IVF treatment improves or deteriorates the quality of the endometrium.

Materials and methods

Patients

In the study group (IVF group), endometrial tissue was obtained from 20 women participating in an IVF/ICSI program. These 20 women had no embryo transfer (ET). ET was omitted due to total fertilization failure (9 with male factor infertility, 7 with idiopathic infertility, 3 with tubal factor infertility, 1 with polycystic ovary syndrome). In 1 case no embryos were transferred due to severe ovarian hyperstimulation syndrome. In 16 patients IVF was carried out and 4 patients were treated by intracytoplasmic sperm injection (ICSI).

In the control group endometrial biopsies were obtained in a natural cycle from 18 non-pregnant women. These women had a regular cycle. They had a partner with severe male infertility and were recruited from the waiting list for ICSI. One woman was a healthy volunteer with no history of subfertility. Characteristics of both groups are summarized in Table 1.

All women gave informed consent according to the Medical Ethical Review Committee of the University Medical Center Nijmegen.

Table I. Patient characteristics

Variable	IVF patients (n = 20)	Control group (n = 18)
Age (years)	32.7 (3.1)	32.1 (3.0)
FSH (IU/l)	7.2 (4.6-15.7)	6.7 (4.7-8)
Estradiol (pmol/l)	3700 (940-8400)	560 (300-1200)[a]
Progesterone (nmol/l)	170 (54-650)	37 (22-74)[b]
Nulligravida (%)	55	83
Nullipara (%)	60	88

Age is given as means ± SD; FSH, estradiol and progesterone are given as median (range).
[a] $P<0.05$.
[b] $P<0.05$.

IVF procedure

Pituitary down-regulation (long protocol) was achieved using a GnRH analogue (Decapeptyl®; Ferring, the Netherlands). Multiple follicular stimulation was realized by recombinant follicle-stimulating hormone (Puregon®; Organon, Oss, the Netherlands). Thirty-six hours after hCG injection (Pregnyl®; Organon, Oss, the Netherlands) we performed transvaginal oocyte retrieval under systemic analgesia (10 mg oxazepam orally and 1mg alfentanyl, i.v.). The retrieved oocytes were inseminated or ICSI was performed according to the method described by Van Steirteghem and colleagues (26). The morning following injection or insemination fertilization of the oocytes was judged. The luteal phase was supported by three doses of progesterone (200 mg; Progestan®; Nourypharma, Oss, the Netherlands) intravaginally daily from the day of oocyte retrieval until the day of the endometrial biopsy.

Endometrial biopsy

In the IVF group endometrial tissue was obtained by a microcurettage using a Pipelle de Cornier (Prodimed, Neuilly-en-Thelle, France) six days after oocyte retrieval. In the control group an endometrial biopsy was taken seven days after the endogenous luteinising-hormone (LH) surge was detected, tested daily, with a urinary semi-quantative monoclonal-antibody based kit (Clearplan, Unipath Ltd., Bedford, United Kingdom). Median weight of the biopsies was not significantly different between the IVF group (1.1 g) and the control group (0.7 gram).

Of the first three IVF patients and two controls all the collected mate-

rial was used immediately for isolation of uterine mononuclear cells. In the following 17 biopsies in the IVF group and 16 biopsies in the control group a part of the tissue was fixed by formaldehyde and used for histological examination. Endometrial histology was analyzed by Noyes criteria (11) by a single observer who was blinded to the treatment group. In the IVF group the day of oocyte retrieval was considered the day of ovulation (day 0), while in the control group day 0 was defined 24 hours after the LH surge. The endometrium was considered out of phase if there was a discrepancy of three days or more between the observed and expected endometrial maturation, as this was the published margin of error described by the Noyes criteria (11). Glandular-stromal dissociation was observed in one IVF-patient. Peripheral blood samples were drawn immediately before the endometrial biopsy was taken to assess lymphocyte populations and determination of estradiol and progesterone levels.

Isolation of uterine mononuclear cells

Endometrial tissue was suspended in a very small volume of RPMI-1640 with glutamax supplemented with pyruvate containing 100 U/ml penicillin, 100 mg/ml streptomycine (all from Gibco, Paisley, UK) and10% heat inactivated pooled human serum and then disrupted very carefully by mincing between two scalpels. This was performed very thoroughly until the suspension only contained fragments of maximally 0.5 mm. The cell suspension was then filtered through a 70 µm sieve while gently pushing with the back of a plunger. Living mononuclear cells were isolated from the cell suspension by density centrifugation (Lymphoprep, Nycomed, Oslo, Norway) yielding a median of $1.0x10^6$ mononuclear cells per biopsy in the IVF group and $0.5x10^6$ in the control group (p=ns). Median weight of the biopsies was not significantly different between the IVF group (1.1 g) and the control group (0.7 gram). PBMC were isolated from blood samples by density centrifugation (Lymphoprep, Nycomed, Oslo, Norway).

Monoclonal antibodies and flow cytometry

Cells were phenotypically analyzed by a direct one-step triple labeling procedure. The following monoclonal antibodies were used: CD3 (clone UCHT1) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), CD16 (clone DJ130C) conjugated with FITC, CD45 (clone

T29/33) conjugated with PE all from Dako (Glostrup, Denmark), CD56 (clone NKH1) conjugated with PE -cytochrome 5 (CY5), CD158a (clone EB6) conjugated with PE, 158b (clone GL183) conjugated with PE, NKG2A/CD94 (clone Z199) conjugated with PE all from Coulter Immunotech (Marseille, France). The ILT-2 antibody was a kind gift from Dr. López-Botet (Madrid, Spain) and was stained indirectly using a GAM-PE antibody. The samples were run on a Coulter Epics XL Flowcytometer (Beckman Coulter, Fullerton, CA, USA), and 10,000 events were collected based on live leukocyte gating as indicated by propidium iodide (PI, 5 mg/ml) in a forward and side scatter dot plot. Backgating on CD3 and CD56 confirmed that practically all lymphocytes were located in the gate. Isotype matched antibodies were used to define marker settings. Analysis of the data was performed using Coulter Epics Expo 32 software (Beckman Coulter, Fullerton, CA, USA). CD45 was used as common leukocyte marker and T cells were identified as $CD3^+CD56^-$cells, NK cells as $CD56^+CD3^-$ cells and NKT cells as $CD3^+CD56^+$ cells.

Immunohistochemistry

Formalin-fixed and paraffin-embedded endometrial biopsies were used. From 17 IVF patients and 16 controls a four-micrometer thick paraffin section was stained using the monoclonal antibody CD45RB, clone PD7/26 (Dako, Denmark). The tissue sections were stained with the recently developed and highly sensitive, specific and ready to use PowerVision kit (ImmunoLogic, the Netherlands) in a dilution 1:800 in PBS. $CD45^+$ leukocytes were scored by counting 5 randomly selected microscope fields at a magnification of x400. The average number of cells per x400 field is reported. The slides were scored by three investigators blinded to the identity of the samples.

Statistical analysis

NK, NKT, T cell, $CD45^+CD3^-CD56^-$ cell numbers and the NK subsets were expressed as a percentage of the number of CD45+ cells. The Mann-Whitney-U test was used to test for statistical differences in mean percentages of leukocyte populations and FSH value between the IVF and the control group. The *t*-test for independent samples was used, after log-transformation if appropriate, to test for statistical significant differences of mean values of age, estradiol and progesterone levels between

the IVF and control group. All tests were two-tailed with a confidence interval of 95% ($p<0.05$). All tests were performed using the Statistical Package for Social Sciences.

Results

IVF treatment does not result in endometrial advancement

According to expectation, the median levels of estradiol and progesterone in peripheral blood were significantly higher in the IVF group as compared to the control group (3700 pmol/L and 170 nmol/L versus 560 pmol/L and 37 nmol/L, respectively ($p<0.0001$). There was no difference in age, obstetrical history and basal FSH level between the IVF and control group (Table 1).

Endometrial histology can be used as a measure of endometrial quality since extreme endometrial advancement (3 days or more) results in lower pregnancy rates. To determine whether hormone treatment affected endometrial advancement, biopsies were scored according to the criteria of Noyes (11). There was no significant difference in number of patients with endometrial advancement between the IVF group and the control group (37 % and 19%, respectively, Figure 1). Thus, hormone treatment did not affect endometrial advancement.

Hormone treatment shifts the balance away from the cytotoxic $CD56^{dim}CD16^{+}$ population

Another way to define endometrial quality could be by defining the leukocyte composition of the endometrium and more particular NK cell ratios. During implantation $CD56^{bright}$ NK cells are imputed to play an important role while $CD56^{dim}$ NK cells are mostly negatively associated with reproduction. Therefore, we determined absolute numbers of leukocytes in endometrial biopsies and more importantly, we assessed leukocyte subpopulations by flowcytometric analysis as a measure of the total population of leukocytes (i.e. CD45+).

CD45 staining of paraffin sections of endometrial biopsies showed that hormone treatment resulted in higher leukocyte levels (Figure 2). The average number of leukocytes in the IVF group was 5.7 per X400 field compared to 2.7 in the control group ($p<0.05$).

We found that the proportion of the uterine $CD56^{dim}CD16^{+}$ NK cells

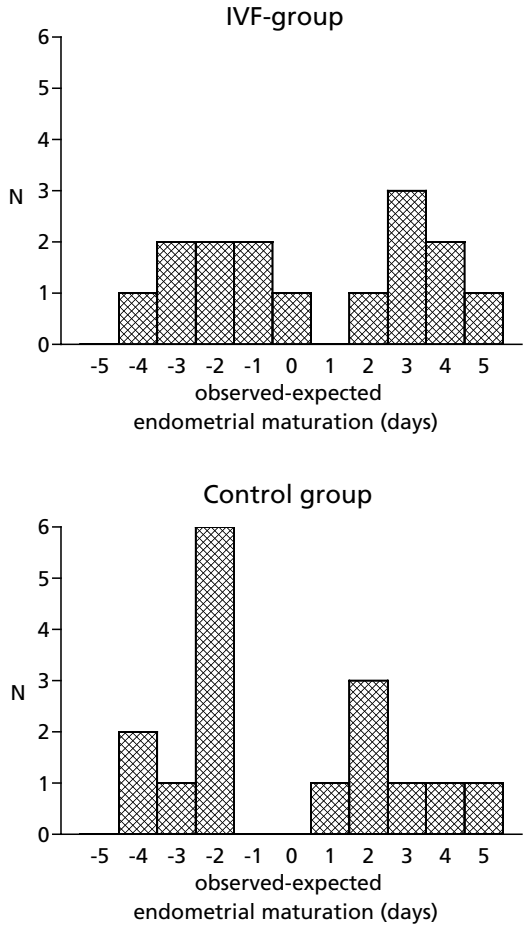


Figure 1. Endometrial maturation. The data are illustrated as a frequency distribution of the endometrial glandular dating expressed as observed (Noyes' criteria) minus expected (calculated from day of ovulation) endometrial maturation in days per patient in the IVF group and the control group.

was significantly lower in IVF patients as compared to the controls (6.9% and 13.7%, respectively, $p<0.01$, Table II, Figure 3). The proportion of $CD56^{bright}$ NK cells ($CD56^{+}/CD16^{-}$) was not different between IVF patients and controls in the uterus (28.8% and 33.5% respectively, Figure 3). These data indicate a shift in the ratio towards the beneficial $CD56^{bright}$ NK cells and away from the harmful $CD56^{dim}$ NK cells.

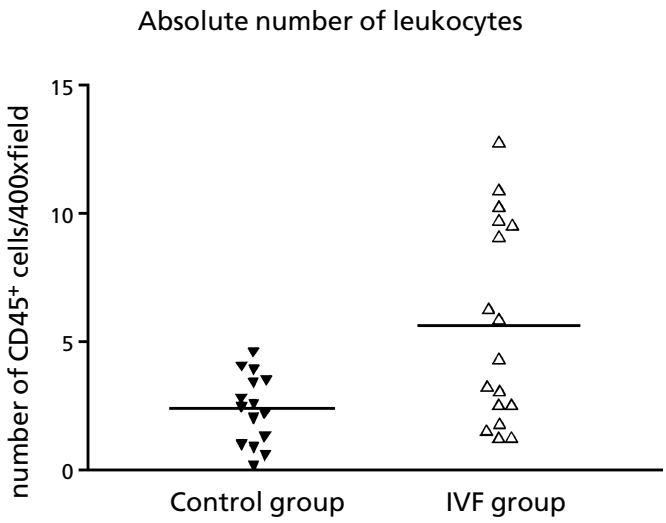


Figure 2. The endometrium of hormone-treated women contains a higher number of leukocytes. The absolute number of $CD45^+$ cells per x400 field was scored per patient by counting five randomly selected microscope fields at a magnification of x400. The average number of cells per x400 field is reported.

Table II. Levels of lymphocyte populations in endometrium and peripheral blood

	Endometrium		Peripheral blood	
Cells (%)	IVF (n = 20)	Control (n = 18)	IVF (n = 15)	Control (n = 16)
T cells ($CD45^+CD3^+CD56^-$)	33.4	36.1	73.3	69.1
NKT cells ($CD45^+CD3^+CD56^+$)	3.3	5.1	4.0	3.7
NK cells				
$CD16^+CD56^{dim}$NK	6.9	13.7[a]	12.3	13.1
$CD16^-CD56^{bright}$NK	28.8	33.5	1.3	0.9
$CD45^+CD3^-CD56^-$	27.6	11.6[b]	9.1	13.2

All values are given as mean percentage ± SD.
[a] $p<0.01$, in endometrium.
[b] $p<0.001$, in endometrium.

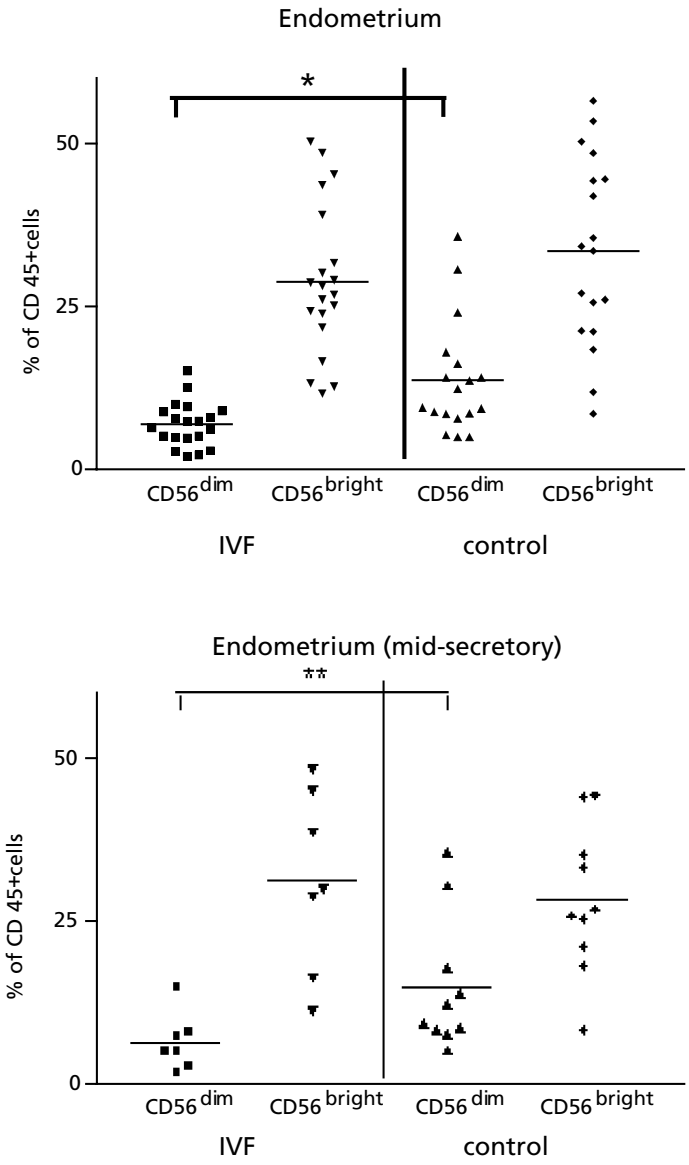


Figure 3. The proportion of cytotoxic $CD56^{dim}$ NK cells is lower in the endometrium of hormone-treated women. NK cell populations in endometrium in all patients (upper panel) and in patients in the mid-secretory phase (lower panel) derived from hormone-treated women (IVF patients) compared to control women. Cells were analyzed by flow cytometry, gating on the living lymphocytes. Data are expressed as percentage of the whole leukocyte population (i.e. $CD45^{+}$). $CD56^{dim}$ = $CD16^{+}/CD56^{+}$; $CD56^{bright}$ = $CD16^{-}/CD56^{++}$. * $P < 0.05$. ** $P < 0.01$. All tests were performed by the Mann-Whitney-*U*-test.

The relative number of uterine T cells remained unaffected by ovarian stimulation (31.7% and 36.1%, respectively). The level of $CD45^{-}CD3^{-}CD56^{-}$ cells, i.e. mainly monocytes and few B-cells was significantly higher in the endometrium of IVF patients as compared to the control group (27,6% and 11.6%, $p<0.001$, Table 11).

Similar results were obtained when the analyses were limited to patients that were shown to be in the mid-secretory phase, seven in the IVF group and 10 in the control group (Figure 1). The percentage $CD56^{bright}$NK cells was significantly lower in the IVF-group as compared to the control group (6.5% and 15.1%, $p<0.01$, Figure 3) while the percentage monocytes was increased (23% and 11%, $p<0.005$). No differences were observed in proportion of $CD56^{bright}$NK cells (21% and 28%), T cells (35% and 40%) and NKT-cells (3.5% and 5.1%) between IVF patients and controls, respectively.

Since the IVF group contained patients with different causes of infertility we also performed a subgroup analyses to exclude that the observed NK shift was a result of a disturbed endometrial function in a subgroup of patients. Similar results, i.e. lower levels of $CD56^{dim}$ NK cells, were obtained when analysing IVF patients with male factor versus controls infertility ($p<0.02$) and patients with idiopathic infertility versus controls ($p<0.02$).

In peripheral blood there is no shift in the $CD56^{dim}/CD56^{bright}$ ratio

Next we determined whether hormone treatment induced a systemic shift in the $CD56^{dim}/CD56^{bright}$ ratio or whether this was a purely local phenomenon i.e restricted to the endometrium. Therefore, the leukocyte composition of peripheral blood of IVF patients and control women was compared.

First, as expected, in these women the leukocyte composition of peripheral blood was shown to be different from the endometrium. In peripheral blood the NK cell number was relatively low as compared to the T cell number (approximately 14% and 70%, respectively) and most of the NK cells were of the $CD56^{dim}$ phenotype (90%).

Second, when comparing peripheral blood samples of IVF patients and controls, no differences in the proportion of $CD56^{dim}$ or $CD56^{bright}$ NK cells were observed (Figure 4 and Table 11). Neither were there differences in T cell numbers, nor in number of $CD45^{+}CD3^{-}CD56^{-}$ cells. Thus, the effect of hormone treatment on the shift in the $CD56^{dim}/CD56^{bright}$

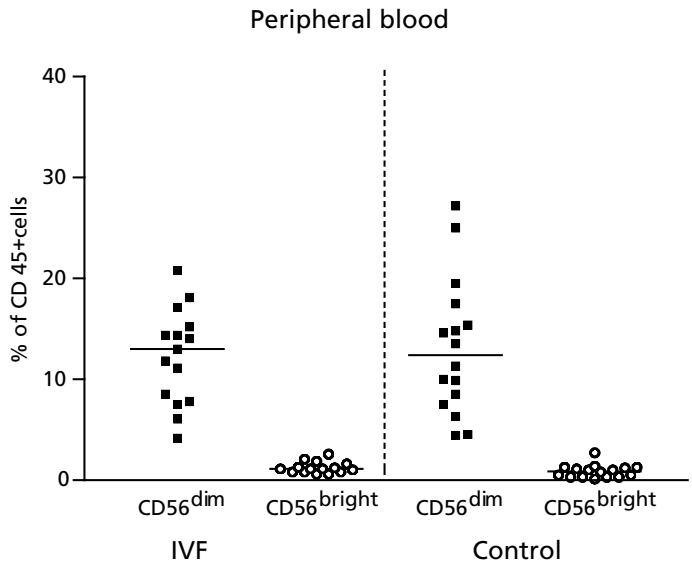


Figure 4. In peripheral blood there is no shift in the $CD56^{dim}/CD56^{bright}$ ratio. NK cell populations in peripheral blood derived from hormone-treated women (IVF patients) compared to control women. Cells were analyzed by flow cytometry, gating on the living lymphocytes. Data are expressed as percentage of the whole leukocyte population (i.e. $CD45^{+}$). $CD56^{dim}$ = $CD16^{+}/CD56^{+}$; $CD56^{bright}$ = $CD16^{-}/CD56^{++}$.

ratio appears limited to the endometrium and is not observed in peripheral blood.

Endometrium contains a unique CD56^{superbright} population

When analyzing the uterine NK cell populations, we observed a double fluorescence peak of CD56 expression within the $CD56^{bright}$ NK cell population. One subset had a similar expression of CD56 as peripheral blood $CD56^{bright}$ NK cells and the second one had a clearly higher expression of the marker CD56 (Figure 5). Based upon this finding, we defined three distinct NK cell populations in the uterus, i.e. $CD56^{dim}$, $CD56^{bright}$ and $CD56^{superbright}$. The $CD56^{superbright}CD16^{-}$ was unique for the uterus and was not found in peripheral blood (Figure 5).

To further characterize whether these uterine $CD56^{superbright}CD16^{-}$ NK cells differed from uterine $CD56^{bright}$ NK cells, we determined the expression of various NK cell receptors that are involved in interactions with HLA molecules on trophoblast cells, namely CD158a and CD158b

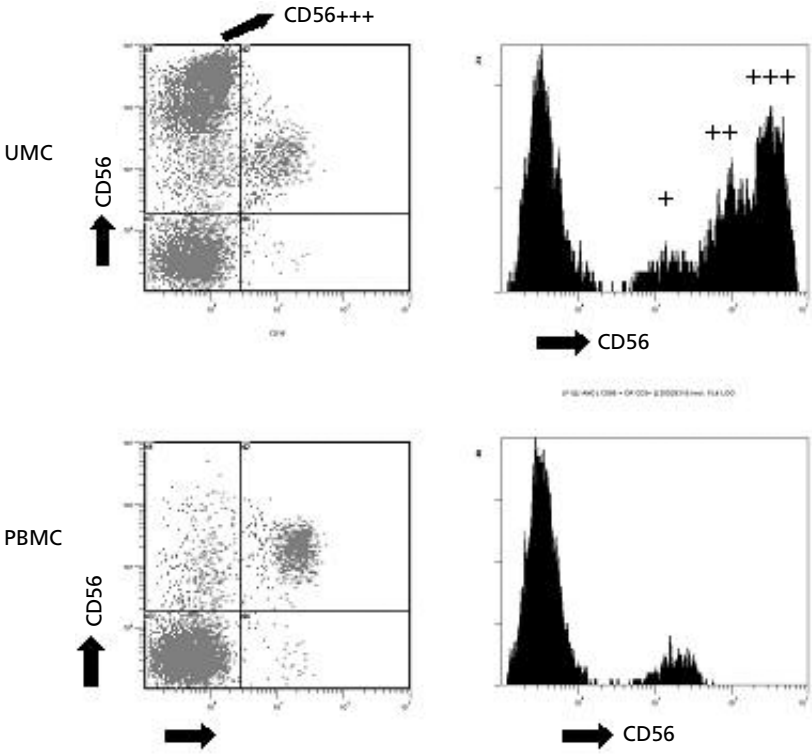


Figure 5. The endometrium contains a population of CD56^{superbright} cells that is not present in peripheral blood. CD56^{dim} is defined as CD16^{+}/CD56^{+}; CD56^{bright} is CD16^{-}/CD56^{++}and CD56^{superbright} is CD16^{-}/CD56^{+++}. UMC = uterine mononuclear cells; PBMC = peripheral blood mononuclear cells.

that bind to HLA-Cw, ILT-2 that binds to HLA-G and NKG2A that binds to HLA-E. The data show, that the CD56^{superbright} cells do indeed differ from CD56^{bright} NK cells with respect to NK cell receptor expression (Figure 6). A significantly higher number of CD56^{superbright} NK cells express CD158a, CD158b, ILT2 and NKG2A as compared to CD56^{bright} NK cells (Figure 7, $p<0.05$). Also, the level of expression of NKG2A was significantly higher in the CD56^{superbright} NK cell population as compared to CD56^{dim} and CD56^{bright} NK cells. There was a trend towards a higher level of CD158a, CD158b and ILT2 expression in the CD56^{superbright} NK cells as compared to the CD56^{bright} NK cells (Figure 7).

In conclusion, many CD56^{superbright} NK cells express NK cell receptors and practically all cells of this subset are NKG2A positive. These cells

were only found in the endometrium and not in peripheral blood. In contrast, only few uterine CD56^{bright} NK cells express the NK cell receptors CD158a, -b and ILT2, although the majority of cells (80%) are NKG2A positive. This pattern is comparable to peripheral blood CD56^{bright} NK cells.

There was no significant difference in CD56^{bright} NK cell number between the IVF and control group (14.8% and 19.7%, respectively). This also applies for the number of CD56^{superbright} NK cells between the IVF and control group (13.8% and 13.8%, Figure 8).

Discussion

A critical event at the onset of a pregnancy is the implantation of the embryo in the uterus. At that time there is believed to be an active dialogue between fetal trophoblast cells and uterine lymphocytes that are present in the endometrium. During IVF-treatment a patient is exposed to supraphysiological levels of sex steroid hormones either directly by administration (progesterone) or indirectly due to multiple follicle growth (estradiol and progesterone). In the current study, we determined whether treatment with exogenous hormones affected the composition of the lymphocyte populations in the endometrium during the window of implantation. We paid special attention to the uterine NK cell subset, since it is thought that NK cells play an important role in human reproduction (27). Our data show that ovarian stimulation leads to a shift in the ratio of CD56^{dim}/ CD56^{bright}CD16^{-} NK cells, i.e. a relative decrease in the cytotoxic CD56^{dim}/CD16+ NK cell population.

From literature it is known, that the CD56^{dim}CD16^{+} NK cell subset is negatively associated with reproductive events (17-20;28). Fukui *et al.* (17) reported higher cytotoxicity and CD56^{dim}CD16^{+} NK cell count in endometrial tissue of IVF patients experiencing implantation failure or an abortion compared to an ongoing pregnancy. In contrast, the CD56^{bright}CD16^{-} NK cell population is associated with successful implantation and placental maturation (13) and is supposed to be crucial at the time of implantation by controlling trophoblast invasion and the production of immunoregulatory cytokines (24;29). An important function of the invading trophoblast cells is vascular transformation of the spiral arteries to provide an adequate blood supply to the fetus and pla-

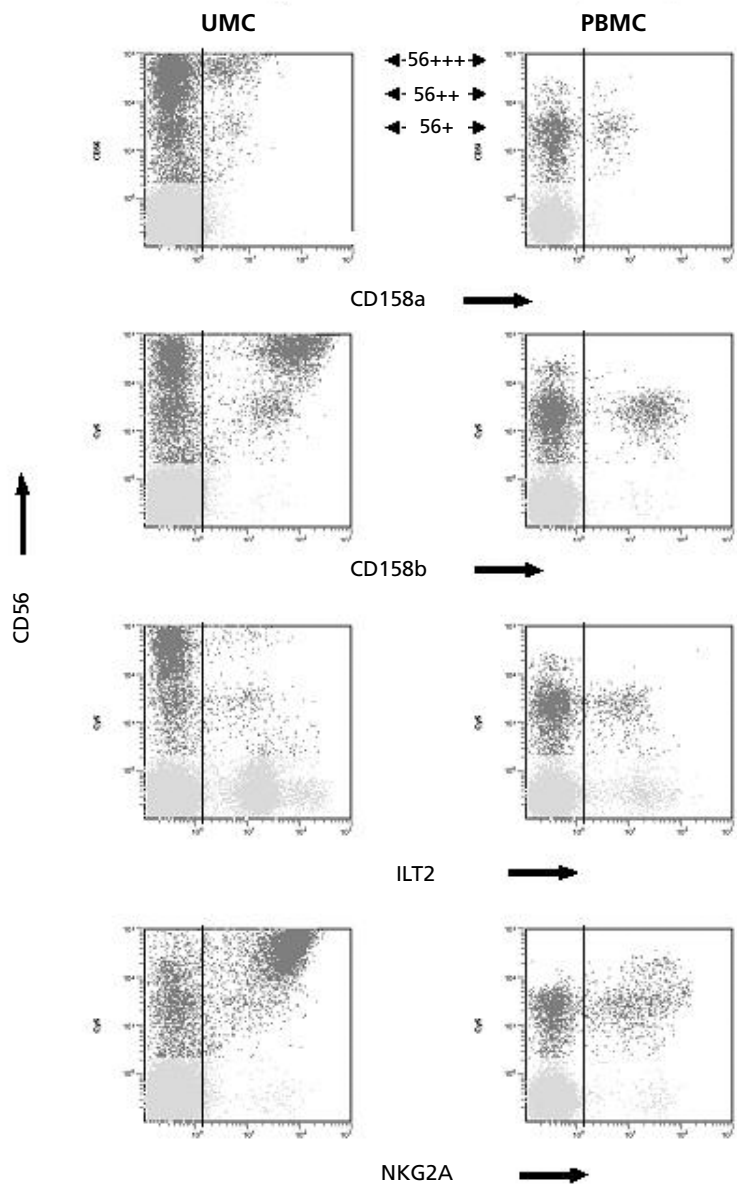


Figure 6. CD56^{superbright} NK cells differ from CD56^{bright} NK cells with respect to number and expression levels of NK cell receptors. Uterine and peripheral blood mononuclear cells were stained with CD56^{-}PC5 and CD3^{-}FITC in combination with CD158a, CD158b, ILT2 or NKG2A labelled with phycoerythrin. Double staining of CD56 and the different NK cell receptor is shown for a single representative example. CD56^{+}, CD56^{++} and CD56^{+++} represent CD56^{dim} NK cells, CD56^{bright} NK cells and CD56^{superbright} NK cells respectively.

centa. Our data suggest that hormone treatment shifts the balance towards the CD56^{bright} cells and away from the cytotoxic CD56^{dim} NK population and would thus positively affect endometrial quality.

The shift in NK cell subpopulations does not appear to be due to differences in endometrial maturation between hormone-treated women and control women. First, we found no significant difference in histological dating between those two groups. Second, when the analyses were limited to women that were in the mid-secretory phase, similar results were obtained, i.e. a decrease in the proportion of CD56^{dim} NK cells.

We discovered even a third subset of NK cells in the endometrium at the time that it is receptive for implantation, which has not been described previously. We showed that, based upon the NK cell marker CD56 and NK cell receptor expression, the CD56^{bright} population could be subdivided into two different populations. The so-called CD56^{superbright} cells, which are CD16 negative, were detected only in the endometrium and not in peripheral blood. Furthermore, in contrast to both uterine and peripheral CD56^{bright} NK cells, a large proportion of CD56^{superbright} NK cells express NK cell receptors for HLA-C and -G molecules that are present on the invading trophoblasts. Practically all CD56^{superbright} express NKG2A at high levels. This latter receptor binds HLA-E, a non-classical class Ib molecule. These features of CD56^{superbright} NK cells suggest different functional properties as compared with CD56^{bright} cells. Preliminary data that we obtained from a single specimen of decidua of a 13-week pregnant woman with a cervix carcinoma indicate that the CD56^{superbright} NK cell population can also be found in the decidua. Maybe it is this CD56^{superbright} subset of the CD56^{bright} NK cells that is responsible for the favorable effects on reproduction as described above.

It has been shown by us as well as others that the absolute number of leukocytes in the endometrium increases under the influence of sex steroids (25). This would suggest that the shift in the CD56^{dim}/CD56^{bright} ratio is caused by specific recruitment of the less cytolytic NK cell population. So far the mechanisms behind this specific recruitment of leukocyte subsets remains uncertain. Estrogen receptors have been detected on NK cells (30), although there is some controversy about this (31). Alternatively, chemokine receptor expression on CD16^{-} cells (32) and on CD56^{bright} cells and monocytes (Drake et al, 2003) might be involved in specific homing to invading extravillous trophoblast cells.

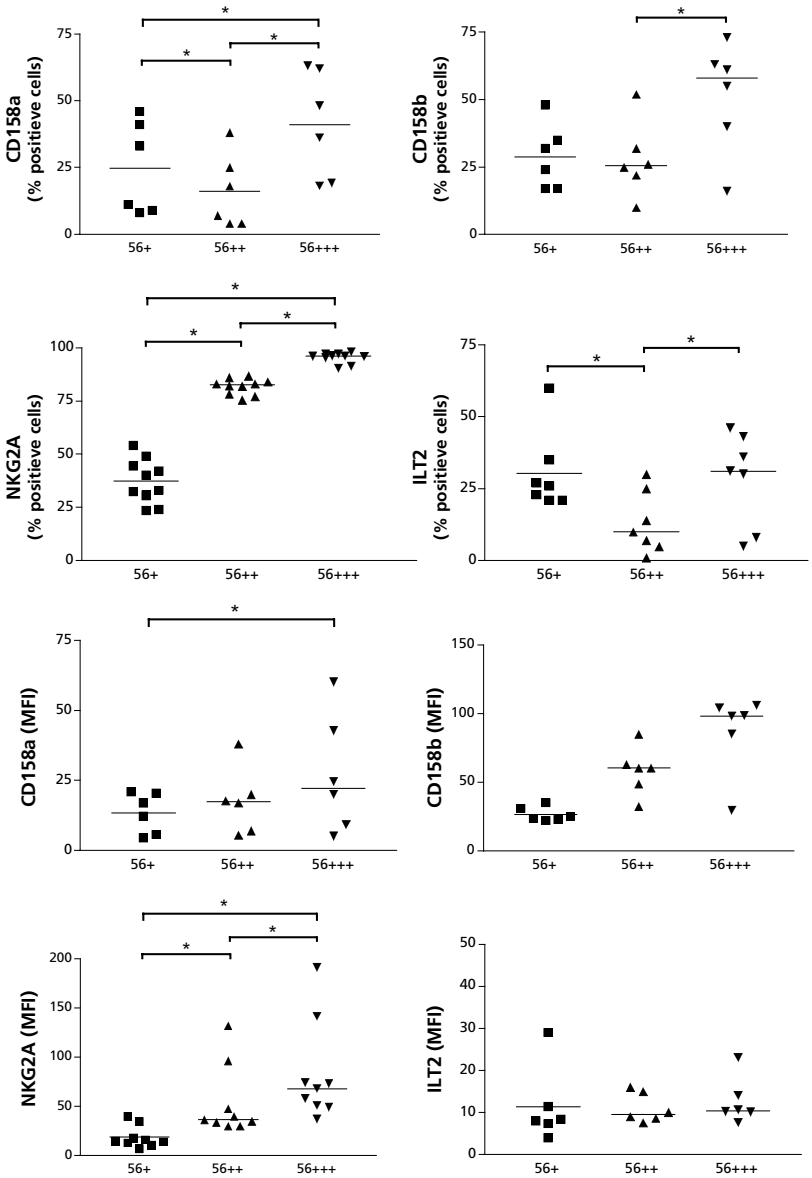


Figure 7. CD56^{superbright} NK cells differ from CD56^{bright} NK cells with respect to number and expression levels of NK cell receptors. Upper panel (four graphs) represents the percentage of NK cells expressing a particular receptor expressed as percentage of the total population of that particular NK cell (CD56^{dim}, CD56^{bright} or CD56^{superbright}). Lower panel (four graphs) represents expression level per NK receptor positive cell (expressed as mean fluorescence intensity). The line represents the mean value. CD56^{dim} is defined as CD16^{+}/CD56^{+}, CD56^{bright} is CD16^{-}/CD56^{++} and CD56^{superbright} is CD16^{-}/CD56^{+++}. Gating for the CD56^{superbright} population was based upon maximal Cy5 signal found in the peripheral blood NK cell population.

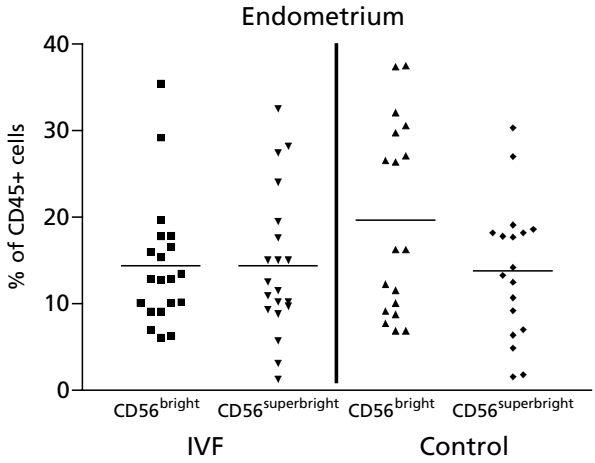


Figure 8. CD56^{superbright} NK cell levels do not differ between hormone-treated women and controls. Cells were analyzed by flow cytometry, gating on the living lymphocytes. Data are expressed as percentage of the whole leukocyte population (i.e. CD45^{+}). CD56^{bright} = CD16^{-}/CD56^{++} and CD56^{superbright} = CD16^{-}/CD56^{+++}. Gating for the CD56^{superbright} population was based upon maximal Cy5 signal found in the peripheral blood NK cell population.

Differential expression of chemokine receptors by human CD56^{bright} and CD56^{dim} NK cells indeed suggests that these subsets may home to different microenvironments in vivo (33).

A feature of endometrial quality is the receptivity determined by histological dating of endometrial tissue by criteria of Noyes et al. (11). Biopsies, in natural as well as in ovarian stimulated cycles, which are out of phase by three days or more, are associated with a reduction in clinical pregnancy rates (5;7;34). In contrast to others (8;9;35), we did not find significantly more cases of endometrial advancement in the IVF group as compared with the control group, although we are aware of the small numbers of biopsies investigated. So, we suppose that in our study the supraphysiological sex steroid concentrations have no deleterious impact upon implantation.

In summary, we conclude that hormonal stimulation for IVF treatment positively affects the CD56^{bright}/ CD56^{dim} ratio of the endometrium during the window of implantation by a relative decrease in the cytotoxic CD56^{dim}CD16^{+} NK cell number. Further studies are needed to investigate the relationship between NK cell subset composition of endometrium and pregnancy rates after ovarian stimulation in order to improve stimulation protocols in assisted reproductive technology.

References

1. Paulson RJ, Sauer MV, Lobo RA. Embryo implantation after human in vitro fertilization: importance of endometrial receptivity. Fertil.Steril. 1990;53(5):870-4.
2. Simon C, Cano F, Valbuena D, Remohi J, Pellicer A. Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. Hum.Reprod. 1995;10(9):2432-7.
3. Chenette PE, Sauer MV, Paulson RJ. Very high serum estradiol levels are not detrimental to clinical outcome of in vitro fertilization. Fertil.Steril. 1990;54(5):858-63.
4. Macklon NS, Fauser BC. Impact of ovarian hyperstimulation on the luteal phase. J.Reprod.Fertil.Suppl 2000;55:101-8.
5. Klentzeris LD, Bulmer JN, Warren A, Morrison L, Li TC, Cooke ID. Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. Am.J.Obstet.Gynecol. 1992;167(3):667-74.
6. Ubaldi F, Bourgain C, Tournaye H, Smitz J, Van Steirteghem A, Devroey P. Endometrial evaluation by aspiration biopsy on the day of oocyte retrieval in the embryo transfer cycles in patients with serum progesterone rise during the follicular phase. Fertil.Steril. 1997;67(3):521-6.
7. Kolibianakis E, Bourgain C, Albano C, Osmanagaoglu K, Smitz J, Van Steirteghem A et al. Effect of ovarian stimulation with recombinant follicle-stimulating hormone, gonadotropin releasing hormone antagonists, and human chorionic gonadotropin on endometrial maturation on the day of oocyte pick-up. Fertil.Steril. 2002;78(5):1025-9.
8. Garcia JE, Acosta AA, Hsiu JG, Jones HW, Jr. Advanced endometrial maturation after ovulation induction with human menopausal gonadotropin/human chorionic gonadotropin for in vitro fertilization. Fertil.Steril. 1984;41(1):31-5.
9. Chetkowski RJ, Kiltz RJ, Salyer WR. In premature luteinization, progesterone induces secretory transformation of the endometrium without impairment of embryo viability. Fertil.Steril. 1997;68(2):292-7.
10. Santoro N, Goldsmith LT, Heller D, Illsley N, McGovern P, Molina C et al. Luteal progesterone relates to histological endometrial maturation in fertile women. J.Clin.Endocrinol.Metab 2000;85(11):4207-11.

11. Noyes, R. W., hertig, A. T., and Rock, J. Dating the Endometrial Biopsy. Fertil.Steril. 1(1), 3-25. 1-1-1950.
12. Loke YW, King A. Immunology of human placental implantation: clinical implications of our current understanding. Mol.Med.Today 1997;3(4):153-9.
13. King A. Uterine leukocytes and decidualization. Hum.Reprod.Update. 2000;6(1):28-36.
14. Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K et al. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. J.Clin.Endocrinol.Metab 2001;86(4):1823-34.
15. Jacobs R, Hintzen G, Kemper A, Beul K, Kempf S, Behrens G et al. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur.J.Immunol. 2001;31(10):3121-7.
16. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22(11):633-40.
17. Fukui A, Fujii S, Yamaguchi E, Kimura H, Sato S, Saito Y. Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization. Am.J.Reprod.Immunol. 1999;41(6):413-22.
18. Beer AE, Kwak JY, Ruiz JE. Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles. Am.J.Reprod.Immunol. 1996;35(4):376-82.
19. Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veerhoek M, Joosten I. Peripheral Natural Killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. Hum.Reprod. 2000;15(5):1163-9.
20. Ntrivalas EI, Kwak-Kim JY, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD et al. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. Hum.Reprod. 2001;16(5):855-61.
21. King A, Allan DS, Bowen M, Powis SJ, Joseph S, Verma S et al. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. Eur.J.Immunol. 2000;30(6):1623-31.
22. Hunt JS, Petroff MG, Burnett TG. Uterine leukocytes: key players in pregnancy. Semin.Cell Dev.Biol. 2000;11(2):127-37.
23. Kanai T, Fujii T, Unno N, Yamashita T, Hyodo H, Miki A et al. Human leukocyte antigen-G-expressing cells differently modulate the release of cytokines from mononuclear cells present in the decidua versus peripheral blood. Am.J.Reprod.Immunol. 2001;45(2):94-9.

24. Rieger L, Hofmeister V, Probe C, Dietl J, Weiss EH, Steck T et al. Th1- and Th2-like cytokine production by first trimester decidual large granular lymphocytes is influenced by HLA-G and HLA-E. Mol.Hum.Reprod. 2002;8(3):255-61.
25. DeLoia JA, Stewart-Akers AM, Brekosky J, Kubik CJ. Effects of exogenous estrogen on uterine leukocyte recruitment. Fertil.Steril. 2002;77(3):548-54.
26. Van Steirteghem A, Tournaye H, Van der Elst J, Verheyen G, Liebaers I, Devroey P. Intracytoplasmic sperm injection three years after the birth of the first ICSI child. Hum.Reprod. 1995;10(10):2527-8.
27. Moffett-King A. Natural killer cells and pregnancy. Nat.Rev.Immunol. 2002;2(9):656-63.
28. Kwak JY, Kwak FM, Ainbinder SW, Ruiz AM, Beer AE. Elevated peripheral blood natural killer cells are effectively downregulated by immunoglobulin G infusion in women with recurrent spontaneous abortions. Am.J.Reprod.Immunol. 1996;35(4):363-9.
29. King A, Burrows T, Verma S, Hiby S, Loke YW. Human uterine lymphocytes. Hum.Reprod.Update. 1998;4(5):480-5.
30. Henderson TA, Saunders PT, Moffett-King A, Groome NP, Critchley HO. Steroid receptor expression in uterine natural killer cells. J.Clin.Endocrinol.Metab 2003;88(1):440-9.
31. Stewart JA, Bulmer JN, Murdoch AP. Endometrial leucocytes: expression of steroid hormone receptors. J.Clin.Pathol. 1998;51(2):121-6.
32. Hanna J, Wald O, Goldman-Wohl D, Prus D, Markel G, Gazit R et al. CXCL12 expression by invasive trophoblasts induces the specific migration of CD16 negative human natural killer cells. Blood 2003;102(5):1569-77.
33. Robertson MJ. Role of chemokines in the biology of natural killer cells. J.Leukoc.Biol. 2002;71(2):173-83.
34. Davies MC, Anderson MC, Mason BA, Jacobs HS. Oocyte donation: the role of endometrial receptivity. Hum.Reprod. 1990;5(7):862-9.
35. Ben Nun I, Jaffe R, Fejgin MD, Beyth Y. Therapeutic maturation of endometrium in in vitro fertilization and embryo transfer. Fertil.Steril. 1992;57(5):953-62.

This study was in part supported by Organon NV, the Netherlands.

7

Membrane-bound HLA-G activates proliferation and interferon-γ production by uterine natural killer cells

Arnold van der Meer[1]
Marieke H.G.M. Lukassen[2]
Marie-José C. van Lierop[3]
Frank Wijnands[3]
Sietse Mosselman[3]
Didi D.M. Braat[2]
Irma Joosten[1]

Departments of Bloodtransfusion and Transplantation Immunology[1] and Obstetrics and Gynaecology[2], University Medical Centre Nijmegen, Nijmegen, the Netherlands.

Department of Pharmacology[3], NV Organon, Oss, the Netherlands.

Molecular Human Reproduction 2004; 10:189-195

Abstract

The expression of HLA-G by invading trophoblasts suggests a role for this molecule in embryo implantation. Putative targets for HLA-G are the uterine NK cells (uNK) that are abundantly present at the time of implantation. Since NK cells are potent producers of a variety of cytokines, interaction with HLA-G may result in the production of cytokines involved in trophoblast differentiation or tissue remodelling. In the present study we investigated the effect of membrane-bound HLA-G (mHLA-G) on the uterine mononuclear cell population (UMC) as a whole and on uNK cells in particular by measuring proliferation and cytokine production (IFN-γ/ VEGF/ LIF/ IL-3). Uterine cells were isolated from endometrium of non-pregnant woman at the time that the endometrium is thought to be receptive to implantation, and then co-cultured with HLA-class I^-/HLA-class II^+ 721.221 B-LCL cells transfected with mHLA-G. HLA-G suppressed the alloproliferative response of unfractionated UMC to 721.221 cells. Also, IFN-γ and IL-3 production was strongly reduced. In contrast, purified uNK cells were stimulated by mHLA-G. Proliferation as well as IFN-γ production was increased after co-culture with mHLA-G transfected 721.221 cells. HLA-G stimulated VEGF production by UMC as well as purified uNK cells. LIF-levels were below the detection level of our ELISA. In conclusion, our data show that mHLA-G stimulates proliferation and cytokine production by NK cells, while downmodulating the response of unfractionated UMC.

Introduction

Implantation of the embryo in the uterus is a critical event in human pregnancy. During this event, foetal trophoblast cells attach to and invade the maternal decidua, altering the tissue in such a way that an appropriate blood flow to the placenta can develop. A typical characteristic of the invading trophoblasts is the expression of the non-classical MHC molecule HLA-G, besides HLA-C and HLA-E (1;2).

HLA-G, in contrast to the classical HLA class II molecules, has a restricted tissue distribution and low polymorphism. Expression is limited to thymic epithelial cells and to the foetal-maternal interface, where it is expressed by invasive trophoblast cells. Also, it is found to be present in amniotic fluid. Recent data suggest that HLA-G expression is also induced in peripheral organs/tissues under certain pathological conditions (3;4). Seven splice variants of HLA-G exist, four membrane-bound forms and three soluble forms. Although there still is debate on the expression and function of the different isoforms, it is well established that the full-length membrane bound and soluble forms, HLA-G1 and -G5 respectively, are expressed, stable and functionally active (5-7). So far, three different receptors have been identified that can putatively interact with HLA-G, i.e., ILT2 (8;9), ILT4 (8) and KIR2DL4 (10;11). These receptors are present on the different leukocyte populations that are present in the endometrium, i.e. NK cells (KIR2DL4 and ILT2), subsets of T cells (ILT2), B-cells (ILT2 and ILT4) and monomyelocytic cells (ILT2 and ILT4).

There is in vitro evidence that HLA-G has an immunomodulatory effect on peripheral lymphocytes. The membrane-bound form has been shown to protect cells from lysis by NK cells (12), it inhibits the allo-proliferative response in a mixed lymphocyte culture (13;14) and affects cytokine production (15).

In the endometrium the main population of leukocytes present are the NK cells. Their number gradually increases during the menstrual cycle, peaking at the time of implantation. When pregnancy occurs, the NK cells remain in the decidua and come into close contact with the invading trophoblast. Their abundant presence at the time of implantation and the expression of receptors for HLA-G suggest a role for NK cells by modulating implantation by interaction with HLA-G, present on the invading trophoblast.

Two functions of HLA-G in modulating uterine NK cells have been postulated. First, they might protect trophoblasts from NK cell mediated lysis (12). More recent data however suggest that this protection appears to be largely independent of HLA class I expression (2;16). Therefore, the second option is more likely, which is to modulate production of cytokines and angiogenic factors by uterine NK cells in order to alter trophoblast invasion and differentiation or tissue remodeling (17). This would suggest a more finely tuned modulatory function for HLA-G rather than a strictly inhibitory.

Cytokines that are produced by NK cells and/or T cells and that might play a role in implantation are IFN-γ, vascular endothelial growth factor (VEGF), interleukin 3 (IL-3) and leukaemia inhibitory factor (LIF). During implantation IFN-γ may play a dual role. It has been shown that a localized production of IFN-γ by uterine NK cells is necessary for vascular modification and decidual integrity and thereby contributes to normalcy of pregnancy in mice (18). A more generalized IFN-γ response however is likely detrimental, since this was found to be associated with pregnancy disorders (19;20). VEGF is an important growth factor involved in angiogenesis by affecting endothelial cells (21) and was shown to be present in endometrium as well as in the decidua during gestation (22;23). Leukaemia inhibitory factor (LIF) has been shown to be an essential cytokine for implantation in mice (24). In humans LIF has been shown to be produced by uNK cells as well T cells (25). IL-3 has been shown to affect trophoblast implantation and development in vitro (26).

In the present study we investigated the effect of membrane-bound HLA-G on production of these various cytokines and proliferation of the uterine mononuclear cell population as a whole, and on uterine NK cells in particular. Since we were interested in cytokine production at the time of implantation, uterine cells were isolated from the endometrium of non-pregnant woman during the phase that the endometrium is receptive for implantation, i.e. seven days after the LH surge.

Materials and methods

Patients

Endometrial tissue was obtained from healthy non-pregnant women or women participating in an IVF program who experienced total fertiliza-

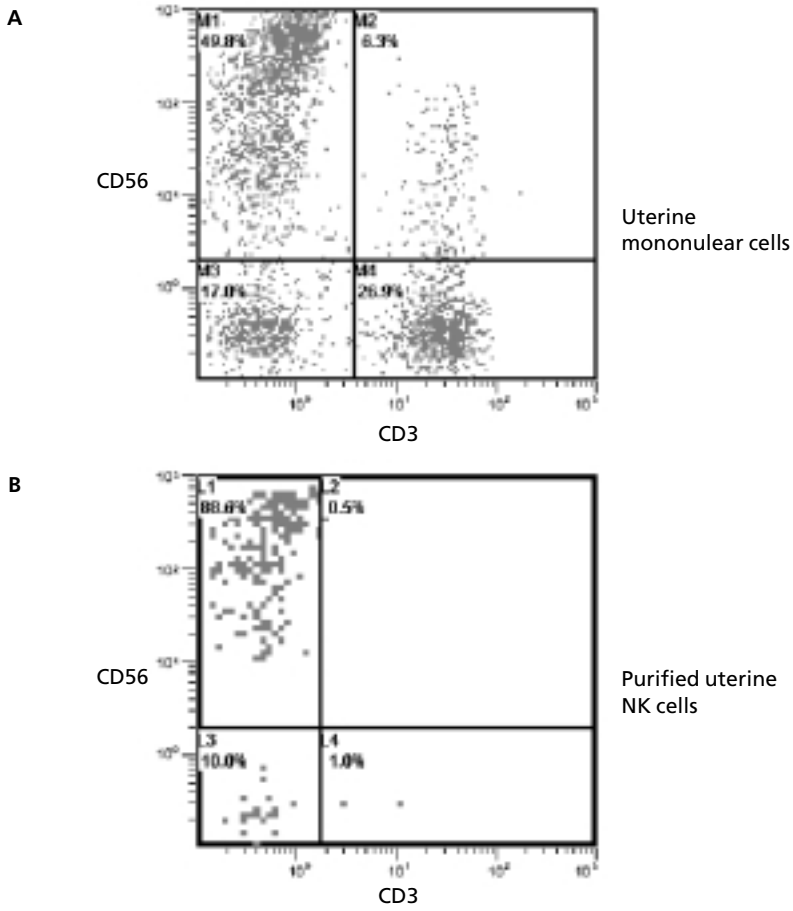


Figure 1. Flow cytometric analysis of natural killer (NK) cells (CD56) and T cells (CD3) in the uterine mononuclear cell fraction directly after isolation from the endometrium biopsy (**A**) and after NK cell purification (**B**). Analysis was performed on the living $CD45^+$ cell population.

tion failure by a microcurettage using a pipelle de cornier (Prodimed, Reilly-en-Thelle, France) at day seven after the LH surge in a natural cycle or six days after oocyte retrieval in case of IVF treatment. All 13 women (8 IVF and 5 control) gave informed consent according to the Medical Ethical Review Committee of the University Medical Center Nijmegen.

Isolation of uterine mononuclear cells and uterine NK cells

Endometrial tissue (median weight 1 g, 0.2 - 1.2 g) was mechanically disrupted by mincing between two scalpels and then filtered through a

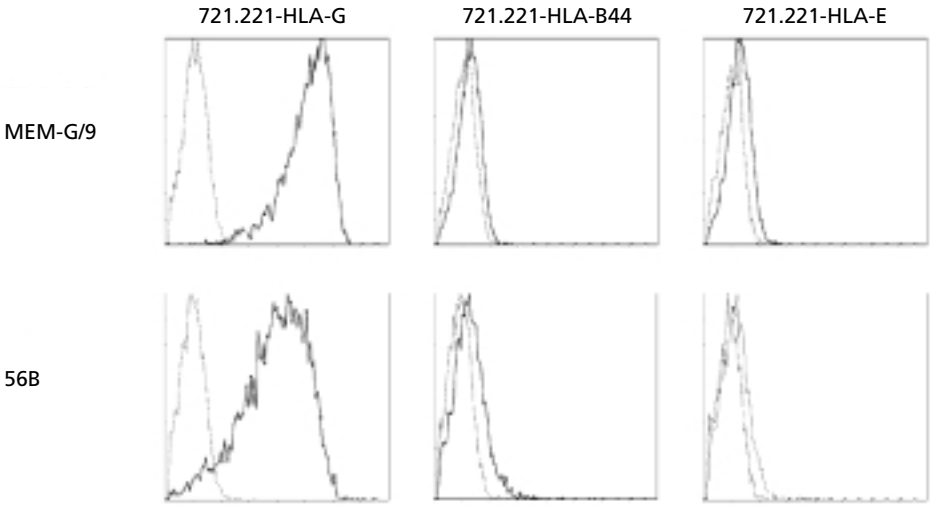


Figure 2. Expression of membrane-bound HLA-G in 721.221 cells was confirmed by flow cytometry using the HLA-G specific monoclonal antibody MEM-G/9 and 56B. HLA-G specificity of the 56B was confirmed by staining HLA-B44 transfected 721.221 cells and HLA-E transfected 721.221 cells. The grey line represents the isotype control, the dark line represents MEM-G/9 or 56B staining.

sieve. Mononuclear cells were isolated from the cell suspension by density centrifugation (Lymphoprep, Nycomed, Oslo, Norway) yielding a median of $1.5x10^6$ mononuclear cells per biopsy (ranging from 0.5 to 3.4 $x10^6$). The cellular composition was analyzed by flow cytometry.

Uterine NK cells were purified from the UMC population by negative selection (a cocktail of anti-CD3, CD4, CD19 and CD33 antibodies) using magnetic cell sorting (MACS) according to the manufacturers protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). Purity of the NK cell fraction was tested by flow cytometry and varied between 80% and 95%. In all cases the percentage of T cells was below 2%. One representative example is shown in Figure 1.

Flow cytometry

$1x10^5$ cells were stained directly using a combination of CD3-FITC/CD45-PE/CD56-Cy5 or CD16-FITC/CD3-PE/CD56-Cy5. Except CD56-Cy5 (Coulter Immunotech, Marseille, France) all antibodies were purchased from Dako (Glostrup, Denmark). The samples were run on a Coulter Epics XL Flowcytometer (Beckman Coulter, Fullerton, CA), and

10,000 events were collected based on live lymphocyte cell gating as indicated by Propidium Iodide (PI, 5 mg/ml) staining. Isotype matched antibodies, usually below background staining, were used to define marker settings. Analysis of the data was performed using Coulter Epics Expo 32 software (Beckman Coulter, Fullerton, CA, USA).

Monoclonal antibodies and cell lines

The HLA-G specific antibody 56B was generated by immunization of Balb/c mice with a peptide corresponding to amino acids 138 to 158 of the α2 domain of HLA-G (27). HLA-G specificity was shown by Western blot and flow cytometry. Flow cytometric analysis of 721.221 cells transfected with HLA-G, HLA-B44 or HLA-E confirmed HLA-G specificity and showed no cross-reactivity with HLA-E (Figure 2). Similar results were obtained with K562 cells transfected with full-length HLA-G or HLA-E (kindly provided by Prof.Dr. E.H. Weiss, Munich, Germany, data not shown). The HLA-G specific antibody MEM-G/9 was obtained from Exbio (Prague, Czech Republic). The HLA-E specific antibody DT9 was a kind gift from Dr. V. Braud (Valbonne, France).

The HLA-A,-B,-C and -G deficient B-cell line 721.221 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). 721.221 cells were stable transfected by electroporation with the full-length transcript of HLA-G1 inserted in the pNGVI-vector. HLA-G expression was confirmed by flow cytometry using the HLA-G specific monoclonal antibody MEM-G/9 or 56B as shown in figure 2. HLA-B44 transfected 721.221 cells were a kind gift from Dr. H. Dolstra (Nijmegen, the Netherlands). HLA-G1(E_{neg})721.221 cells were a kind gift from Dr.M. López-Botet (Madrid, Spain). These cells have been transfected with an HLA-G1 cDNA that has a mutated signal sequence that does not allow binding to HLA-E and thus prevents co-expression of HLA-E (28). HLA-E 721.221 cells contains the HLA-E*0101 gene and the HLA-B8 signal sequence and were a kind gift from Dr. V. Braud (Valbonne, France). Cells were continuously kept in culture in RPMI-1640 with glutamax supplemented with pyruvate containing 100 U/ml penicillin, 100 mg/ml streptomycin (all from Gibco, Paisley, UK),10% heat inactivated foetal calf's serum (FCS) and geneticin (1μg/ml, Gibco, Paisley, UK). Cells were washed extensively before use in the co-cultures and HLA-G or -B44 expression was checked for by FCM analysis using the monoclonal antibody w6/32.

Co-cultures

UMC or uterine NK cells were plated at 5x10^4 cells/well in triplicate in 96-wells U-bottom microtiter plates (Greiner, Frickenhausen, Germany) in the presence of irradiated (100 Gy) 721.221 cells or HLA-G transfected 721.221 cells (5x10^4 cells/well) or HLA-B44 transfected or HLA-G1(E_{neg}) transfected 721.221. As a control cells were plated in culture medium alone (RPMI-1640 with glutamax supplemented with pyruvate containing 100 U/ml penicillin, 100 mg/ml streptomycine (all from Gibco, Paisley, UK) and10% heat inactivated pooled human serum). In case of blocking HLA-G, the anti-HLA-G Moab 56B was added to the wells at a concentration of 5 µg/ml. An isotype matched irrelevant Moab was used as negative control. Analysis of the kinetics showed a peak in proliferation and IFN-γ production around day 5. Therefore, after 5 days incubation at 37°C in a humidified atmosphere containing 5% CO_2, supernatant was harvested for cytokine measurements and subsequently 0.5 µCi [^{3}H]TdR was added to each well. The plates were harvested the following day (day 6) using a Micromate 196 harvesting device (Canberra, Meriden, CT, USA) and counted in the Packard Matrix 96 Direct Beta counter (Canberra). The results are presented as the mean ± SD from triplicate wells.

Enzyme-linked immunosorbent assay (ELISA)

Cytokines were measured in cell culture supernatants. IFN-γ (Pelikine Compact human ELISA kit, CLB, Amsterdam, the Netherlands), IL-3, VEGF (both from Biosource International, Camarillo, USA,) and LIF (R&D systems, Minneapolis, USA) were measured by sandwich ELISA. The VEGF ELISA only detects the VEGF-165 form and not PIGF. The assays were performed according to the supplier's manual. The results were measured photometrically at 450nm using an ELISA plate reader (Titertek Multiskan MCC/340). VEGF data were corrected for background production of VEGF by irradiated 721.221 cells or 721.221 cells transfected with mHLA-G. Data are mean of a triplicate.

Statistical analysis

Differences between groups were analyzed for significance ($p<0.05$) by a two-sided students t-test or, in case of three sample groups, by a one-way anova.

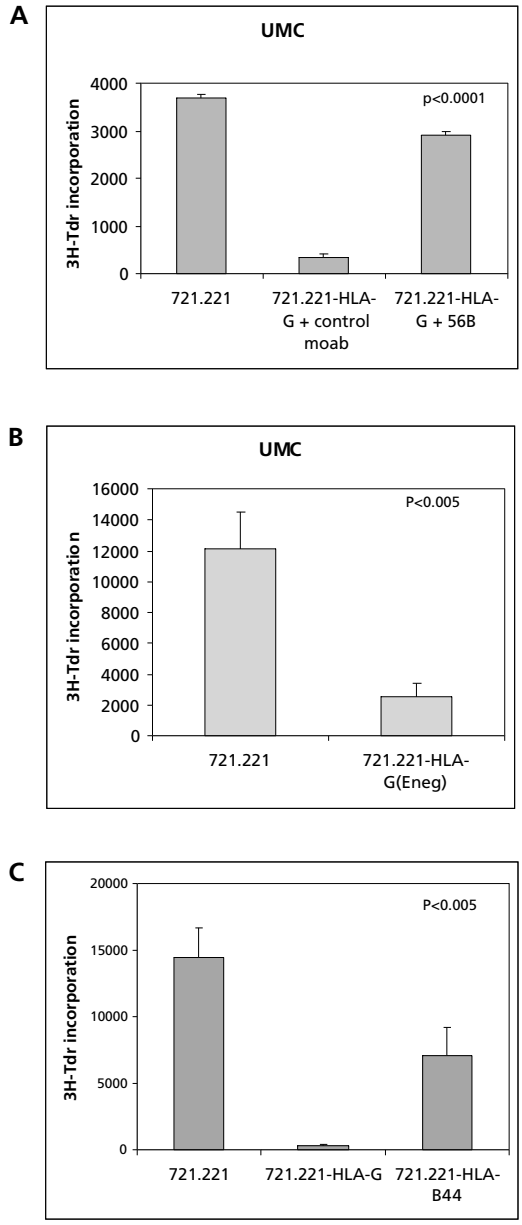


Figure 3. Proliferative response of uterine mononuclear cells (UMC) after co-culture with irradiated 721.221 cells or 721.221 cells transfected with membrane-bound HLA-G (**A**), HLA-G(E_{neg}) (**B**) or HLA-B44 (**C**). Results show the mean of triplicates and are expressed as mean ± SD and are a single representative experiment out of four separate experiments.

Results

Membrane-bound HLA-G stimulates proliferation of uterine NK cells

To study the effect of membrane-bound HLA-G on the proliferation of uterine lymphocytes and in particular uterine NK cells, 721.221 cells transfected with HLA-G1 were used as stimulator cells in a mixed lymphocyte culture. 721.221 cells are HLA-A and -B negative, but HLA class II positive and can thus function as allogeneic stimulator cells. The data show that expression of mHLA-G suppressed the allo-induced proliferation of UMC consisting of NK cells, T cells, B-cells and monocytes/macrophages (Figure 3a, $p<0.0001$). In four independent experiments this inhibition varied from 46 up to 100% as compared to cultures with non-transfected 721.221 cells. The inhibitory effect of HLA-G could at least partly be reversed by the addition of the anti-HLA-G monoclonal antibody 56B to the cultures (Figure 3a). Since the 721.221-HLA-G transfected cells express both HLA-G and HLA-E at the cell surface, we further confirmed HLA-G specificity of the effect by using the HLA-G(E_{neg}) 721.221 cell-line. This cell-line was negative for HLA-E expression as was shown by staining with HLA-E specific antibody DT9 (data not shown). Again, proliferation of UMC was inhibited by the HLA-G expressing cells up to 80% in four independent experiments (Figure 3b, $p<0.005$), clearly showing that the inhibition was induced by HLA-G.

Furthermore, the inhibitory effect appeared not broadly class I specific, since the response to HLA-B44 transfected 721.221 cells was not strongly inhibited (Figure 3c), although in different experiments the strength of the response was variable.

In contrast to the overall inhibitory effect observed for the unfractionated population, mHLA-G stimulated proliferation of purified uterine NK cells (Figure 4a, $p<0.005$). While purified uterine NK cells hardly proliferate on stimulation with untransfected 721.221 cells, expression of mHLA-G resulted in enhanced proliferation of this population. Again a similar effect was observed when the HLA-G(E_{neg}) 721.221 cells were used (Figure 4b, $p<0.05$).

Membrane bound HLA-G stimulates IFN-γ production by uterine NK cells

Next, we addressed the question of whether mHLA-G can affect cytokine production by UMC or purified uNK cells. Supernatants of co-cultures set up with UMC or purified uterine NK cells and mHLA-G

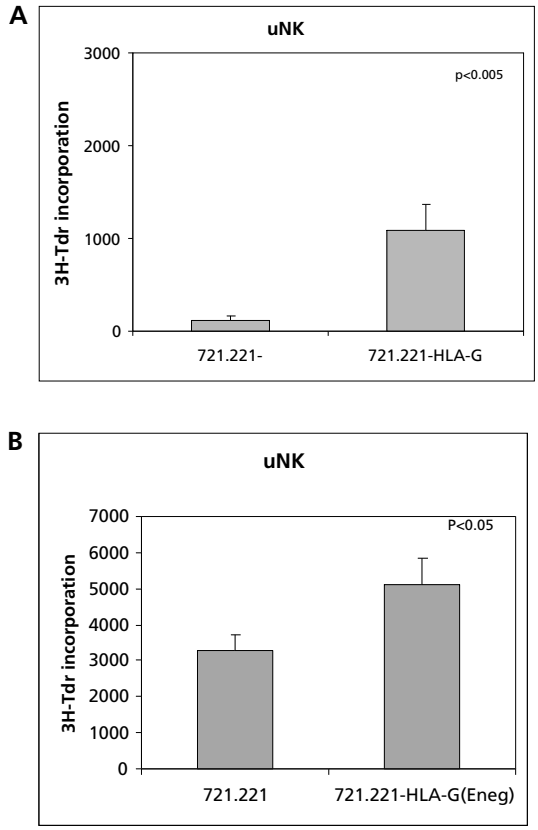


Figure 4. Proliferative response of purified uterine natural killer cells after co-culture with irradiated 721.221 cells or 721.221 cells transfected with membrane-bound HLA-G (**A**) or HLA-G(E_{neg}) (**B**). Results show the mean of triplicates expressed as mean ± SD and are a single representative experiment out of three (HLA-G) or two [HLA-G(E_{neg})] separate experiments.

transfected 721.221 cells were analyzed by sandwich-ELISA for IFN-γ levels. Membrane-bound HLA-G significantly inhibited the 721.221 induced IFN-γ production of UMC (Figure 5a, $p<0.05$, percentage inhibition ranging from 27 to 100 % in four different experiments). Co-culture with HLA-B44 transfected 721.221 cells also lead to a slight reduction in IFN-γ production, although this was never as outspoken as for HLA-G. Notably and in line with the proliferation data, when purified uterine NK cells were used, an increase in IFN-γ was observed (Figure 5b, $p<0.05$). For both UMC and purified uNK cells, similar data were obtained when using the HLA-G(E_{neg}) 721.221 cells (data not shown).

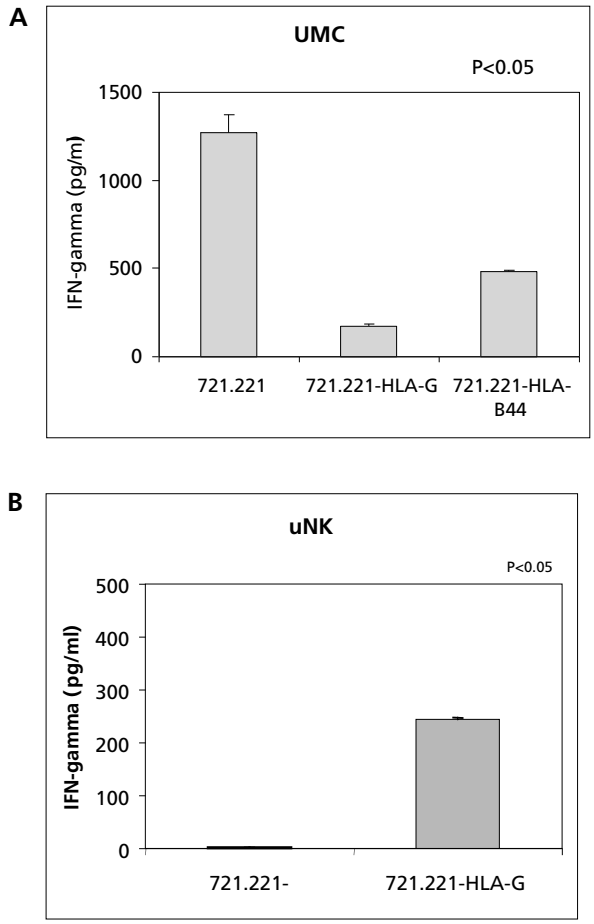


Figure 5. Interferon-γ concentrations in the culture medium of uterine mononuclear cells (UMC) (**A**) or purified uterine natural killer (uNK) cells (**B**) after co-culture with irradiated 721.221 cells or 721.221 cells transfected with membrane-bound HLA-G or HLA-B44. The data shown are the results from a single representative experiment out of four (UMC) or three (uNK) separate experiments.

Membrane bound HLA-G stimulates VEGF production

Besides IFN-γ, the effect of mHLA-G was also evaluated for three other cytokines that potentially play a role in implantation and placentation and are produced by T cells and/or NK cells, i.e. VEGF, IL-3, and LIF.

Regarding VEGF, our data show that UMC (Figure 6a, $p<0.005$) as well as purified uterine NK cells (Figure 6b, $p<0.05$) produce increased levels of VEGF upon co-culture with mHLA-G transfected 721.221 cells

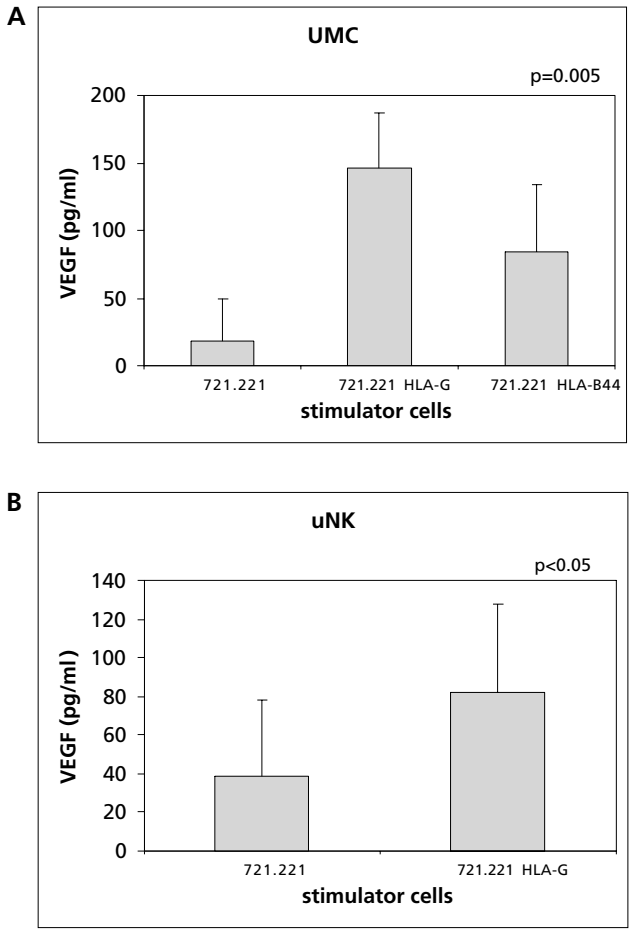


Figure 6. Vascular endothelial growth factor (VEGF) concentrations in the culture medium of uterine mononuclear cells (UMC) (**A**) or purified uterine natural killer (uNK) cells (**B**) after co-culture with irradiated 721.221 cells or 721.221 cells transfected with membrane-bound HLA-G or HLA-B44. The data shown are the results from a single experiment representative of three (UMC and uNK) separate experiments.

as compared to the untransfected 721.221 cells. Co-culture with HLA-B44 transfected 721.221 cells did not significantly affect VEGF production (Figure 6a).

In response to untransfected 721.221 cells UMC produced only very low levels of IL-3 (Figure 7). Co-culture with mHLA-G transfected 721.221 cells in all cases lead to a further decrease in IL-3 production although the reduction in levels did not reach statistical significance (Fig-

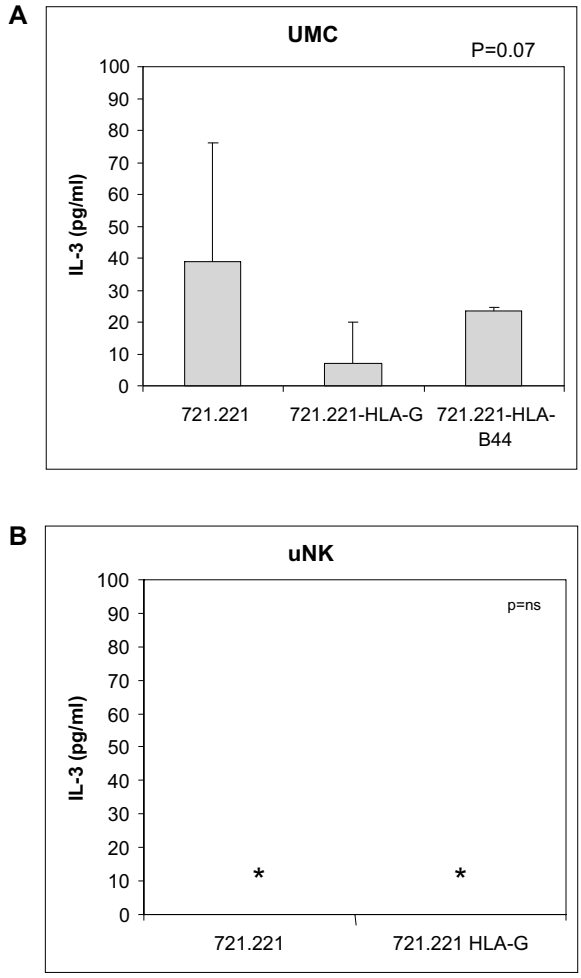


Figure 7. Interleukin-3 (IL-3) concentrations in the culture medium of uterine mononuclear cells (UMC) (**A**) or purified uterine natural killer (uNK) cells (**B**) after co-culture with irradiated 721.221 cells or 721.221 cells transfected with membrane-bound HLA-G or HLA-B44. The data shown are the results from a representative experiment out of four separate experiments. * Below threshold.

ure 7a, p = 0.07). No IL-3 was found in co-cultures with purified uterine NK cells (Figure 7b). Again, similar data were obtained when using the HLA-G(E_{neg}) 721.221 transfectants ($p<0.05$, data not shown) and no significant decrease was observed in co-cultures with HLA-B44 transfected 721.221 cells (Figure 7a).

With respect to LIF, neither UMC nor purified uterine NK cells produced detectable levels of LIF upon stimulation with 721.221 cells or HLA-G transfected 721.221 cells.

Discussion

In this study, we have shown that membrane-bound HLA-G promotes proliferation and IFN-γ production of purified uterine NK cells derived from non-pregnant endometrium at the mid-luteal phase (day LH +7). Thus, our data reveal for the first time that mHLA-G can indeed stimulate specific functions of uterine NK cells that may positively contribute to implantation and decidualization. Studies on uterine lymphocytes so far only pointed towards a role for mHLA-G in protection of the semiallogeneic foetus from an attack by maternal NK and T cells. This was achieved by inhibition of NK cell cytotoxicity (12) and downmodulation of Th1 type cytokine production (29;30). Upregulation of the Th2 response by mHLA-G was found in peripheral lymphocytes, but was not observed for uterine lymphocytes (29).

The increased IFN-γ production by uterine NK cells challenges the Th1/Th2 paradigm as proposed by Wegman et al. (1993), stating that Th1-like cytokines are detrimental for successful pregnancy (19). In line with our data, several other groups have already shown the presence of both IFN-γ encoding mRNA (31;32) and IFN-γ protein (33) in uNK cells derived from pregnant women. Furthermore, receptors for IFN-γ are present on all first trimester cytotrophoblast populations, macrophages and vascular endothelial cells in the human decidua (33). In mice it has been shown that IFN-γ produced by uNK cells promotes the initiation of uterine vascular modification and decidual integrity and thereby contributes to the normalcy of pregnancy (18;34). The finding that uNK cells produce IFN-γ in response to mHLA-G lends support to the idea that IFN-γ may play a role during the first stages of human pregnancy when HLA-G expressing invading trophoblasts come in contact with uterine NK cells. The role of IFN-γ may be very localized, effective within a restricted time frame and dependent on the expression levels, or as Chaouat stated with respect to the involvement of cytokines in pregnancy "sequential windows and extreme complexity mixed with very precise timing and tuning" (35). A candidate receptor on uterine NK cells

responsible for the upregulation of IFN-γ production is KIR2DL4 (10;11). Triggering of this HLA-G binding receptor on resting NK cells has been shown to induce an increased IFN-γ production without affecting lytic function (36).

Because of the extremely limited material we were not able to study the effect of 721.221 HLA-G transfectants on purified uterine T cells. However, it should be appreciated that in case of unfractionated UMC, the proliferation measured, is the net result of the proliferation of T cells and NK-cells. Using peripheral blood mononuclear cells it has been shown that the strong proliferative response of CD4+ T cells to 721.221 cells is inhibited by mHLA-G (14). Purified uterine NK cells show only limited proliferation. In case of UMC the stimulatory effect of mHLA-G on uterine NK cells is probably masked by the strong inhibition of a vigorous uterine T cell response on the allogeneic HLA class II^+ 721.221 cells. This remains to be confirmed by using uterine T cells.

The exact mechanism by which HLA-G induces proliferation of uterine NK cells remains speculative. So far two receptors have been identified that can bind to HLA-G and that are present on (uterine) NK cells, i.e. ILT2 and KIR2DL4. It remains to be determined whether the effect is induced directly via either of these receptors or that a yet unknown receptor plays a role in this effect. Furthermore, autocrine production of cytokines that affect NK cell proliferation may play a role. Decidual NK cells proliferate strongly on IL-15 and this cytokine can be produced by these cells themselves (37). Another important cytokine that plays a role in the induction of NK cell maturation, proliferation and IFN-γ production is IL-21 (38;39). However, it is not clear yet whether uterine NK cells can produce IL-21.

Interestingly unfractionated UMC, including T cells, were inhibited in their IFN-γ production when co-cultured with mHLA-G transfected 721.221 cells. Apparently a generalized IFN-γ response in the uterus should be avoided and HLA-G protects against a too strong response. This down modulation of IFN-γ production by mHLA-G has also been found by others when using unfractionated uterine leukocytes derived from pregnant endometrium (29) as well as peripheral blood mononuclear cells (13-15). This indicates that the effects observed for membrane-bound HLA-G are not solely due to intrinsic properties of uterine lymphocytes but also extend to peripheral lymphocytes and lymphocytes that have already been into contact with foetal cells. Be that as it may, it

cannot be excluded that differences exist at the single cell level or in the modulation of other cytokines than the few we have measured.

In our study we utilized mononuclear cells derived from non-pregnant endometrium at the time that it is receptive for implantation. Most other studies on the interaction between HLA-G and uterine cells have used cells from decidual tissue from elective abortions (2;12;29;30). It has already been shown that decidual NK cells express different activation markers and adhesion molecules as compared to those from non-pregnant endometrium (40). Consequently, it is well conceivable that also the functional capacities of cells derived from non-pregnant endometrium differ since they have not yet been into contact with the invading trophoblasts or have been affected by the altered hormone and cytokine levels that occur during pregnancy.

HLA-G specificity was shown by addition of anti-HLA-G monoclonal antibody to the co-culture of UMC and HLA-G transfected 721.221 cells, leading to reduction of the inhibitory effect of mHLA-G. To further exclude an effect of HLA-E we also used the HLA-G(E^{neg}) transfected 721.221 cells. Furthermore, the finding that proliferation and cytokine production of UMC was not inhibited by HLA-B44 expression on 721.221 cells indicates that the affect is not a broad characteristic of (classical) class II molecules. Nevertheless, this does not rule out the possibility that HLA-E plays a role in the uterus in modulation of the response. The leader peptide of HLA-G is capable of binding to HLA-E, leading to stabilization and surface expression of HLA-E in HLA-G transfected 721.221 cells. Although the receptor for HLA-E (CD94/NKG2) is present on practically all uterine NK cells, recent data indicated that HLA-E expressed on K562 cells does not affect IFN-γ production of decidual NK cells (30). Very little is known so far on the effect of HLA-E on T cells, although it has been shown that HLA-E can be recognized through the ab T cell receptor (41). The fact that the HLA-G specific monoclonal antibody could not fully restore the response may indicate a role for HLA-E in the effect on T cells. The HLA-G transfected 721.221 cells mimic the in vivo situation in the sense that also on trophoblast cells HLA-G and HLA-E are expressed simultaneously (2). It is most likely that the combination of HLA-G and HLA-E loaded with HLA-G derived nonamer peptide, provides the unique signals for modulating local uterine immune responses.

In conclusion, our data suggest an active role for mHLA-G in modu-

lating uterine NK cells that are present in the endometrium at the time that it is receptive for implantation. This indicates that mHLA-G is instrumental in active fine-tuning of the local immune response in order to promote successful implantation.

References

1. King A, Boocock C, Sharkey AM, Gardner L, Beretta A, Siccardi AG et al. Evidence for the expression of HLA A-C class II mRNA and protein by human first trimester trophoblast. J.Immunol. 1996;156(6):2068-76.
2. King A, Allan DS, Bowen M, Powis SJ, Joseph S, Verma S et al. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. Eur.J.Immunol. 2000;30(6):1623-31.
3. Lila N, Carpentier A, Amrein C, Khalil-Daher I, Dausset J, Carosella ED. Implication of HLA-G molecule in heart-graft acceptance. Lancet 2000;355(9221):2138.
4. Aractingi S, Briand N, Le Danff C, Viguier M, Bachelez H, Michel L et al. HLA-G and NK receptor are expressed in psoriatic skin: a possible pathway for regulating infiltrating T cells? Am.J.Pathol. 2001;159(1):71-7.
5. Mallet V, Proll J, Solier C, Aguerre-Girr M, DeRossi M, Loke YW et al. The full length HLA-G1 and no other alternative form of HLA-G is expressed at the cell surface of transfected cells. Hum.Immunol. 2000;61(3):212-24.
6. Bainbridge DR, Ellis SA, Sargent IL. The short forms of HLA-G are unlikely to play a role in pregnancy because they are not expressed at the cell surface. J.Reprod.Immunol. 2000;47(1):1-16.
7. Riteau B, Rouas-Freiss N, Menier C, Paul P, Dausset J, Carosella ED. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. J.Immunol. 2001;166(8):5018-26.
8. Allan DS, Colonna M, Lanier LL, Churakova TD, Abrams JS, Ellis SA et al. Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. J.Exp.Med. 1999;189(7):1149-56.
9. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J et al. A common inhibitory receptor for major histocompatibility complex class II molecules on human lymphoid and myelomonocytic cells [see comments]. J.Exp.Med. 1997;186(11):1809-18.
10. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. J.Exp.Med. 1999;189(7):1093-100.
11. Ponte M, Cantoni C, Biassoni R, Tradori CA, Bentivoglio G, Vitale C et al. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire

p49, an HLA-G1-specific receptor. Proc.Natl.Acad.Sci.U.S.A. 1999;96(10):5674-9.
12. Rouas Freiss N, Goncalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc.Natl.Acad.Sci.U.S.A. 1997;94(21):11520-5.
13. Riteau B, Menier C, Khalil-Daher I, Sedlik C, Dausset J, Rouas-Freiss N et al. HLA-G inhibits the allogeneic proliferative response. J.Reprod.Immunol. 1999;43(2):203-11.
14. Bainbridge DR, Ellis SA, Sargent IL. HLA-G suppresses proliferation of CD4(+) T-lymphocytes. J.Reprod.Immunol. 2000;48(1):17-26.
15. Maejima M, Fujii T, Kozuma S, Okai T, Shibata Y, Taketani Y. Presence of HLA-G-expressing cells modulates the ability of peripheral blood mononuclear cells to release cytokines. Am.J.Reprod.Immunol. 1997;38(2):79-82.
16. Avril T, Jarousseau AC, Watier H, Boucraut J, Le Bouteiller P, Bardos P et al. Trophoblast cell line resistance to NK lysis mainly involves an HLA class I-independent mechanism. J.Immunol. 1999;162(10):5902-9.
17. Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K et al. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. J.Clin.Endocrinol.Metab. 2001;86(4):1823-34.
18. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. J.Exp.Med. 2000;192(2):259-69.
19. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol.Today 1993;14(7):353-6.
20. Raghupathy R. Th1-type immunity is incompatible with successful pregnancy [see comments]. Immunol.Today 1997;18(10):478-82.
21. Abulafia O, Sherer DM. Angiogenesis of the endometrium. Obstet.Gynecol. 1999;94(1):148-53.
22. Moller B, Rasmussen C, Lindblom B, Olovsson M. Expression of the angiogenic growth factors VEGF, FGF-2, EGF and their receptors in normal human endometrium during the menstrual cycle. Mol.Hum.Reprod. 2001;7(1):65-72.
23. Clark DE, Smith SK, Licence D, Evans AL, Charnock-Jones DS. Comparison of expression patterns for placenta growth factor, vascular

endothelial growth factor (VEGF), VEGF-B and VEGF-C in the human placenta throughout gestation. J.Endocrinol. 1998;159(3):459-67.
24. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 1992;359(6390):76-9.
25. Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S. Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nat.Med. 1998;4(9):1020-4.
26. Di Simone N, Caliandro D, Castellani R, Ferrazzani S, Caruso A. Interleukin-3 and human trophoblast: in vitro explanations for the effect of interleukin in patients with antiphospholipid antibody syndrome. Fertil. Steril. 2000;73(6):1194-200.
27. van Lierop MJ, Wijnands F, Loke YW, Emmer PM, Lukassen HGM, Braat DDM et al. Detection of HLA-G by a specific sandwich ELISA using monoclonal antibodies G233 and 56B. Mol.Hum.Reprod. 2002;8(8):776-84.
28. Navarro F, Llano M, Bellon T, Colonna M, Geraghty DE, Lopez BM. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. Eur.J.Immunol. 1999;29(1):277-83.
29. Kanai T, Fujii T, Unno N, Yamashita T, Hyodo H, Miki A et al. Human leukocyte antigen-G-expressing cells differently modulate the release of cytokines from mononuclear cells present in the decidua versus peripheral blood. Am.J.Reprod.Immunol. 2001;45(2):94-9.
30. Rieger L, Hofmeister V, Probe C, Dietl J, Weiss EH, Steck T et al. Th1- and Th2-like cytokine production by first trimester decidual large granular lymphocytes is influenced by HLA-G and HLA-E. Mol.Hum.Reprod. 2002;8(3):255-61.
31. Jokhi PP, King A, Sharkey AM, Smith SK, Loke YW. Screening for cytokine messenger ribonucleic acids in purified human decidual lymphocyte populations by the reverse-transcriptase polymerase chain reaction. J.Immunol. 1994;153(10):4427-35.
32. Saito S, Nishikawa K, Morii T, Enomoto M, Narita N, Motoyoshi K et al. Cytokine production by CD16-CD56bright natural killer cells in the human early pregnancy decidua. Int.Immunol. 1993;5(5):559-63.
33. Jokhi PP, King A, Loke YW. Cytokine production and cytokine receptor expression by cells of the human first trimester placental-uterine interface. Cytokine. 1997;9(2):126-37.

34. Ashkar AA, Croy BA. Interferon-gamma contributes to the normalcy of murine pregnancy. Biol.Reprod. 1999;61(2):493-502.
35. Chaouat G, Zourbas S, Ostojic S, Lappree-Delage G, Dubanchet S, Ledee N et al. A brief review of recent data on some cytokine expressions at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy. J.Reprod.Immunol. 2002;53(1-2):241-56.
36. Rajagopalan S, Fu J, Long EO. Cutting edge: induction of IFN-gamma production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. J.Immunol. 2001;167(4):1877-81.
37. Verma S, Hiby SE, Loke YW, King A. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. Biol. Reprod. 2000;62(4):959-68.
38. Strengell M, Matikainen S, Siren J, Lehtonen A, Foster D, Julkunen I et al. IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. J.Immunol. 2003;170(11):5464-9.
39. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 2000;408(6808):57-63.
40. Hill JA, Hsia S, Doran DM, Bryans CI. Natural killer cell activity and antibody dependent cell- mediated cytotoxicity in preeclampsia. J.Reprod.Immunol. 1986;9(3):205-12.
41. Garcia P, Llano M, de Heredia AB, Willberg CB, Caparros E, Aparicio P et al. Human T cell receptor-mediated recognition of HLA-E. Eur.J.Immunol. 2002;32(4):936-44.

This study was in part supported by Organon NV, the Netherlands.

8

General Discussion and Summary

Multiple pregnancies are considered to be the most serious complication of IVF. A substantially higher incidence of both maternal and perinatal morbidity and mortality as compared to IVF singleton pregnancies is reported (1). That is why multiple pregnancies require more medical care than singleton pregnancies. Moreover, IVF-pregnancies in itself (singletons and multiples) are at greater risk for obstetric and perinatal complications than spontaneously conceived pregnancies (2-7). In this thesis we studied the effect of single embryo transfer compared with double embryo transfer. Furthermore, we investigated the medical costs of IVF singleton- and twin-pregnancies. We also investigated the results of single embryo transfer in a natural ICSI treatment cycle (without any hormonal stimulation). Because the success of single embryo transfer depends on the implantation rate of the embryo, we studied other, immunological factors that might be important to increase the chance of implantation.

Part I: Clinical Aspects

In the introduction of this thesis (**chapter 1**) we presented in Part I some background information on the medical, social and economical problems related to multiple pregnancies after IVF. A literature update on various solutions to decrease the number of multiple births due to IVF are given (SET, natural cycle IVF, selective embryo reduction). When SET might be put into practice in order to decrease the number of IVF-twins and higher order multiple births, the pregnancy rate could drop slightly as well. The implantation phase seems to be a crucial step in establishing a pregnancy. Our goal was to gain more insight in the immunological processes during implantation, because the fetus is considered to be a semi-allograft that has to be accepted by the maternal immune system in order to survive. In Part II we described the role of the immune system in various anatomical sites of the female reproductive tract (ovary, endometrium, decidua). We explained why we believe that the interaction between maternal NK cells and their subsets on the one hand and the HLA-molecules on fetal trophoblast cells on the other hand play an important role in implantation. Finally, we gave information about the existing evidence of immunological involvement in several pregnancy disorders (IVF-failure, preeclampsia, endometriosis, recurrent abortion).

In **chapter 2** we calculated the difference in costs between singleton and twin IVF-pregnancies from early pregnancy until six weeks after delivery. This was done in a retrospective cost analysis from a health care perspective. The medical cost per twin pregnancy were found to be more than five times higher than per singleton pregnancy, 13,469 euro and 2,550 euro respectively. In the present thesis, we focused on costs for twin and singleton pregnancies from a medical point of view. The reason is that savings on the medical budget due to reduction of the number of twin pregnancies by SET might be spent on other medical treatments, for example IVF treatments. Savings in other compartments, sick leave, travel expenses etc., are not likely to become available for medical treatments (8) and were therefore not included in this study.

It is difficult to extrapolate economic consequences of health care conditions from one region or country to another. Costs related to singleton and twin pregnancies after IVF depend on IVF results, duration of hospitalization, Caesarean section rate, need for neonatal intensive care, costs for hospital care, costs for treatment of handicapped children. These variables may differ from country to country and from year to year.

Obviously, there is an urgent need to improve IVF outcome by reducing the number of multiples. In the Netherlands, as in most North European countries, the transfer of two embryos is now considered as a standard policy, therefore higher order multiple pregnancies (triplets and more) following IVF are an exceptional phenomenon, namely 20 triplets (0.5%) in 2003 (www.nvog.nl). The twin pregnancy rate in our country, however, still was 22% in 2003 (www.nvog.nl). The only way to reduce this number of twin pregnancies tremendously is to increase the percentage of single embryo transfer.

We performed a randomized controlled trial to investigate the effectiveness of two IVF-cycles with single embryo transfer (SET) and one cycle with double embryo transfer (DET) (**chapter 3**). We also calculated the cost-effectiveness of both strategies using the results from **chapter 2**. As we expected the live birth rate after SET to be lower than after DET we compared two consecutive SET cycles with only one DET cycle. The randomized controlled trial was performed in 107 women, aged < 35 years, in their first IVF cycle, with at least one good quality embryo. They were randomized to the SET (n = 54) or DET (n =53) group using a computer-generated random block number table, stratified for primary or

secondary infertility. The cumulative live birth rates per woman randomized of two consecutive cycles of SET (41%, 95% confidence interval (CI): 27-54) versus one cycle of DET (36%, 95% CI 23-49) were comparable. The multiple pregnancy rate, however, was significantly higher: 37% (95% CI 15-59) in the DET and 0% in the in the SET group (p = 0.002). Combining the medical costs of the IVF-treatments (where 1.5 more SET cycles were required to achieve each live birth) and of pregnancies up to six weeks after delivery (see **chapter 2**) the total medical costs per live birth were 13,680 euro for DET and 13,438 euro for SET. So, two cycles with SET are equally effective as one cycle with DET, and the medical costs per live birth up to six weeks after delivery are the same. However, if lifetime costs for severe handicaps are included, more than 7,000 euro per live birth will be saved after implementing SET.

There are a few health economic studies evaluating SET versus DET (9-11), but no cost-effectiveness analysis has been published yet where the effectiveness of SET and DET is based on a randomized controlled trial as in our study. Wolner-Hanssen et al. performed a cost-analysis from a societal perspective and therefore they also included costs of sick leave and (medical and societal) lifetime costs of handicap. They used estimates of hypothetical figures. They found that the total costs for the DET strategy were 4 times higher than for the SET strategy (10). De Sutter and colleagues developed a decision-analytic model to compare SET with DET. Pregnancy rates from both randomized and observational studies were included in this model. They concluded that the SET cost per child born was the same as with DET (11). Gerris et al. performed a real-life prospective health economic study of SET versus DET. All medical costs from IVF-treatment, pregnancy and neonatal period up to 3 months after delivery were prospectively analysed in patients of <38 years of age. This was not a randomized controlled trial, but based upon patient choice for SET or DET, whereas SET was exclusively performed if a high quality embryo was available. They showed that the cost of SET was 7,126 euro and the cost of DET 11,039 euro per liveborn delivery. In the DET group embryo quality was not a selection criterion, therefore both groups were not comparable with respect to chance of pregnancy (9).

Bergh et al. just recently reviewed the results of single embryo transfer (12). The results from seven relevant observational studies indicate that similar pregnancy and delivery rates are achieved with SET and DET. In these studies patients with a good prognosis recieved SET while

poor prognosis patients received DET (9;13-17). Up to now only four randomized controlled trials comparing single embryo transfer (SET) and double embryo transfer (DET) have been published (18-21). These studies all compared the effectiveness of just one SET cycle with one DET cycle. These trials found no significant difference in pregnancy rate between SET and DET. Martikainen et al. and Thurin et al. determined the cumulative live birth rate that included the live birth rate after frozen embryo cycles. It is however odd that they did not include the frozen embryo cycles in the DET group. Moreover, the frozen cycle live birth rate in the SET group was very high (16.4%). When comparing the results of only one fresh-embryo cycle the live birth rate in the SET group was significantly lower than in the DET group, 27.6% and 42.9% respectively. Martikainen and colleagues observed an increase in live birth rate from 30% to 39% after frozen embryo transfers in the SET group and from 40% to 51% in the DET group (19). Van Montfoort et al., Academic Hospital Maastricht, just recently performed an observational study to the ongoing pregnancy rate after SET (n = 55, first cycle) and DET (n = 235, first cycle) in patients younger than 38 years with at least one top quality embryo in the first three treatment IVF cycles. The cumulative pregnancy rate after the first cycle (fresh and frozen cycles in both groups) in the SET group (45%) was significantly higher than in the DET group (34%). Without the contribution of frozen embryo cycles (DET) to the pregnancy rate (12% in the SET and 1% in the DET group) the fresh embryo ongoing pregnancy rate was the same (33% in both groups). The twin pregnancy rate in the DET group was 33% (22).

These results stress the importance of a good cryopreservation programme with low costs to increase the live birth rate of one IVF treatment cycle, especially in case of single embryo transfer. This will reduce the number of second stimulated IVF cycles and it is expected that this will result into a considerable cost reduction. In the Netherlands the overall contribution of frozen cycles to the live birth rate of fresh cycles is only 1.8%. In 11% of all started IVF treatment cycles transfer of frozen embryos could take place (www.nvog.nl). These results are lower than other European countries (23). Two Finnish studies reported a remarkable increase of the pregnancy rate due to frozen embryos in patients treated with elective SET of 18% (15) to 26% (24).

In conclusion, the DET strategy should be abandoned completely in a high-risk group for multiple pregnancies, in favour of the strategy with 2

cycles with SET. Embryo freezing programmes must be optimized in the Netherlands in order to maximize the cumulative live birth rate of SET per started IVF cycle. The reduction of multiple pregnancies will not only save the government a lot of money but most of all the patients and their offspring may be spared from a large range of medical and socio-emotional problems.

Alternative IVF protocols

When SET will be implemented in daily practice to reduce the multiple pregnancy rate, it seems logical that less embryos have to be available for transfer. Furthermore, hormonal stimulation is not without risks like the Ovarian Hyperstimulation Syndrome (OHSS). For these reasons, new mild ovarian stimulation protocols or even IVF in the natural cycle might be (re)considered, although we have to bear in mind that in natural cycle IVF selection of the best quality embryo and the benefit of frozen cycles will be omitted.

In **chapter 4** we investigated the efficacy of ICSI in 29 natural cycles of 25 couples at the department of Obstetrics and Gynaecology of the University Medical Center Nijmegen. To our knowledge this was the first serial and systemic investigation of the efficacy of ICSI in the natural cycle. The inclusion criteria were: female age <37 years, infertility due to severe male factor with indication for ICSI (a total motile sperm count < 1×10^6), regular menstrual cycle between 26-32 days, a baseline follicle-stimulating hormone (FSH) value of <10 IU/L and no female fertility problem diagnosed. Oocyte retrieval was performed in a total of 24 cycles. The oocyte recovery rate per cycle was 58.6% (17/29). In three cases the ICSI procedure could not be performed (in one case due to ejaculatory failure and in two cases due to morphological abnormal oocytes). Finally, ICSI was performed in 14 patients. All the injected oocytes fertilized. In twelve patients embryo transfer was performed (41.4% per cycle). Three patients got pregnant and delivered a healthy child (25% per embryo transfer). Thus, the live birth rate per started cycle was 10.3% (3/29).

The ongoing pregnancy rate of 10.3% per started cycle is satisfactory considering the low burden, low costs and low risks. Moreover, this protocol can be performed on a monthly basis, because there is no need for

the ovaries to recover from hormonal stimulation. Extrapolation of the 10.3% live birth rate after one natural cycle results into 28% cumulative live birth rate after three natural cycles. This is comparable with 32% live birth rate after one stimulated ICSI treatment in women younger than 37 years of age in our setting in 2000 (data not published). So, in the same time span the pregnancy rates are comparable. In our opinion natural cycle ICSI can be an alternative for stimulated ICSI cycles in women younger than 37 years of age and with a regular menstrual cycle.

Although natural cycle IVF and ICSI is a patient-friendly, low-risk and low-cost procedure, the major drawback seems to be the high cancellation rate. Pelinck et al. described in a systematic review on the effectiveness of natural cycle IVF a cancellation rate before oocyte retrieval of 28.9%. Embryo transfer was performed in only 45.5% of the 1800 cycles. The ongoing pregnancy rate was 7.2% per cycle and 15.8% per embryo transfer (25). The same group investigated at the University Hospital Groningen the efficacy of a modified approach of natural cycle IVF (26). In this study a GnRH-antagonist was used to prevent a LH-surge, thus minimizing the cancellation rate. They aimed at monofollicular growth to reduce the risk of multiple pregnancies and ovarian hyperstimulation syndrome. The GnRH-antagonist was started and given daily at a follicle size of 14 mm, together with 150 IU recombinant FSH (rFSH) to prevent aberrant follicle growth. In total 50 patients underwent 119 cycles, with a maximum of three cycles. The cancellation rate before oocyte retrieval was 12.6% (15/119) and in only 43.7% of the cycles embryo transfer was performed (52/119). The ongoing pregnancy rate per cycle was 14.3%. The cumulative ongoing pregnancy rate after three cycles was 34%. Although the cumulative pregnancy rate per cycle is good, the cancellation rate is still rather high. Also the cost-effectiveness should be measured. It seems worthwhile to study the cumulative pregnancy rate after a maximum of nine cycles of *unmodified* natural cycle IVF/ICSI with the so called *modified* natural cycle IVF/ICSI to compare both approaches.

Mild stimulation protocols might anticipate the problem of a high cancellation rate, while less redundant oocytes and embryos will be produced. Hohmann et al. compared a mild ovarian stimulation protocol (150 IU rFSH on cycle day 5 + GnRH antagonist from follicle size of 14 mm) with the standard long GnRH agonist protocol with a fixed dose of 150 IU rFSH. In this study a maximum of two embryos were transferred. The mean number of embryos obtained after the mild stimulation proto-

col was three, whereas the standard long GnRH agonist protocol yielded four embryos. Despite the fact that more cycles were cancelled before oocyte retrieval in the mild stimulation protocol (37%) as compared with the standard long GnRH agonist protocol (16%), the ongoing pregnancy rate per cycle were similar (16%, 8/49 and 18%, 8/45, respectively) (27). The twin pregnancy rate was 38% in both groups. However, a shorter stimulation period and a 27% reduction in exogenous rFSH dose were observed in the mild stimulation protocol. A returning problem of the natural and mild stimulation IVF protocols is the high cancellation rate. Hojgaard et al. evaluated the patient attitude towards mild stimulation protocols (clomiphene citrate), including natural cycle IVF, and the standard long protocol for IVF. They conclude that the majority of patients seem to accept the drawbacks of mild stimulation protocols in terms of cycle cancellation and accordingly the necessity of more treatment cycles against the advantages of such protocols in terms of few side-effects, simplicity and short duration (28). In **chapter 4** we noticed the same positive attitude of our patients towards natural cycle ICSI.

Extrapolation of SET strategy to national level in the Netherlands

It is interesting to extrapolate the cost-effectiveness of the SET strategy to the entire population at local level (Nijmegen) and even on national level. The overall live birth rate, the multiple pregnancy rate and the medical costs (including lifetime costs for handicapped singleton and twin children) after the implementation of SET are important parameters to determine.

The medical costs can be divided into mean costs of one IVF treatment cycle (2,532 euro, **chapter 3**), mean costs of IVF pregnancy up to 6 weeks postpartum per singleton live birth (2,550 euro, **chapter 2**) and per twin live birth (13,469 euro, **chapter 2**) and mean lifetime costs of severe handicapped children per singleton (1,489 euro, **chapter 3**) and twin live birth (20,477 euro, **chapter 3**).

To extrapolate the results of our SET-transfer strategy to the entire IVF/ICSI population in our clinic we estimated the effect of SET during one cycle in the total IVF population in the year 2001 and 2002 (n=1788). Of all first treatment cycles (n=789) 41 percent met our inclusion criteria for SET (326/789). In this group with a high risk on multiples 37% got pregnant with a twin pregnancy rate of 36%. The remaining patients

were excluded for SET and received DET (age > 34 years, , poor quality embryos) or SET because of the fact that only one embryo was available. In this group with a low risk on multiples the pregnancy rate was 14% of which 12.5% twin pregnancies. The overall pregnancy rate was 23% and the twinning rate 27%. If all the couples that met the inclusion criteria would have had SET, with a live birth rate of 26% (see **chapter 3**), the overall pregnancy rate would have dropped from 23% to 19%. The overall twin pregnancy rate would have dropped from 27% to 5%. So, a dramatic fall in twin pregnancies can be accomplished at the cost of a small drop in the overall pregnancy rate after implementing single cycle SET in patients undergoing their first treatment cycle, who are less than 35 years of age, and had at least one excellent or good quality embryo during transfer. If the results of frozen IVF cycles were included in this analysis, this final conclusion would have been even more pronounced .

The next step is the calculation of total costs before and after the implementation of SET in the first cycle in our clinic. The total costs are the costs of the IVF cycles *(n x $C_{IVF\text{-}cycle}$)* plus the costs of the IVF pregnancies up to 6 weeks postpartum together with the lifetime costs of handicapped children, per singleton live birth *($C_{preg\text{-}sing}$ + $C_{hand\text{-}sing}$)* and per twin live birth *($C_{preg\text{-}twin}$ + $C_{hand\text{-}twin}$)*.

Using the formulas shown in appendix a, the total costs for 789 first IVF cycles *before* the implementation of SET in Nijmegen are the costs of IVF cycles (2.0 million euro) plus the costs of IVF-pregnancies up to 6 weeks postpartum together with lifetime costs for handicapped children (2.2 million euro), which amounts to a total of 4.2 million euro.

The total costs for 789 first IVF cycles *after* the implementation of SET in Nijmegen are the costs of IVF cycles (2.0 million euro) plus the costs of IVF-pregnancies up to 6 weeks postpartum together with lifetime costs for handicapped children (0.8 million euro), which amounts to a total of 2.8 million euro (appendix a).

Thus, in our IVF clinic the implementation of SET in the first IVF cycle could save us 1.4 million euro per 789 first cycles in a twin prone population at the cost of a small drop in the overall pregnancy rate (from 23% to 19%). This means a reduction of 33% of the total costs.

If we make the assumption that 1.5 more SET cycles are needed to have the same live birth rate as 1 DET cycle (**chapter 3**), the formula of total costs after implementation of SET will be changed. The costs of IVF cycles will become 1.2 times higher; 1.5 x n x P_{SET} + n x P_{DET}. The costs

for the SET group will become 1.5 times higher than for the DET group (appendix b). The total costs after implementing SET will then become 3.4 million euro (i.e. the costs of IVF cycles (2.4 million euro) plus the costs of IVF-pregnancies up to 6 weeks postpartum together with lifetime costs for handicapped children (1.0 million euro)) (appendix b). So, when 1.5 more SET cycles will be performed to bring the pregnancy rate after SET into the same level as after DET, 0.8 million euro could be saved. This results in a 19% reduction of total costs.

In 2001 and 2002 our IVF clinic started 1788 IVF/ICSI treatment cycles, which is 6.2 % of the total number of cycles in the Netherlands (1788/28742) (www.nvog.nl). To estimate the savings after implementation of SET in the first cycle in all clinics in the Netherlands the amounts have to be multiplied by 1/0.062. This means that on national level roughly 22.6 million euro (1/0.062 x 1.4 million euro) can be saved in two years when SET would be implemented for the first IVF cycle. In case 1.5 more SET cycles will be performed to equalize the pregnancy rate, roughly 13 million euro (1/0.062 x 0.8 million euro) can be saved in two years after implementation of SET.

Consequences of the abolition of reimbursement of IVF treatments

In the past in the Netherlands up to three IVF treatment cycles were reimbursed. Since January 2004 the government has abolished this regulation. Nowadays patients have to pay the first out of three IVF cycles themselves. When infertility patients have effected a supplementary insurance for infertility treatment the first IVF cycle will be (partly) reimbursed. This governmental decision might have detrimental health consequences. In general fertility-specialists do not transfer more than two embryos on medical grounds. However, it can be expected that the trend to transfer less than two embryos will stop under pressure from couples who have to pay the first cycle themselves. This explanation is supported by the literature. Decreasing reimbursement strategies result in higher numbers of embryos per transfer (29-31). Multiple pregnancies make DET less cost-effective than SET, especially in couples with a good prognosis, and therefore this measure will lead to higher costs instead of the intended lower costs. Moreover, inadequate or absent health cover-

age for IVF will also provide unequal access and care to some couples with infertility.

It would be better if this governmental measure, to exclude the first IVF cycle from reimbursement, will be replaced by a more cost-effective measure. At short notice, the "Umbrella" study, financed by ZON-MW, will combine the results of the 5 Dutch studies on the efficiency (effectiveness and cost-analysis) of different IVF strategies (Pre-implantation Genetic Screening, SET, natural cycle IVF/ICSI and mild IVF stimulation protocols) with intent to decrease the number of multiple pregnancies. These studies have been performed in the following IVF centra; Groningen, Maastricht, Rotterdam, Utrecht and Nijmegen. In the end, concrete recommendations should follow from these results that are intended for fertility specialists, infertility patients, insurance companies and last but not least the minister of health. Hopefully, the Umbrella study will lead to a better and more efficient strategy for infertility treatment.

Based on the calculation that nationwide implementation of SET in the first cycle of couples with a good prognosis will save 22.6 million euro in two years, we propose to change the reimbursement regulation in a sense that the first three IVF-cycles are reimbursed completely, provided that SET is performed during the first cycle of couples with a good prognosis. We did not calculate the consequences of alternative scenarios, but it can be speculated that for instance the complete reimbursement of a total of six IVF/ICSI treatment cycles per couple with SET will be more cost-effective than the present regulation. It is important to state that frozen embryo transfer(s) are part of the original cycle and should be reimbursed as well, since the use of frozen embryos is an important condition for a successful SET program.

Furthermore, we seriously have to consider the implementation of natural or mildly stimulated IVF/ICSI SET cycles (with or without GnRH antagonists and low dose rFSH) as these cycles are patient-friendly, low-risk and probably low-cost cycles and can be performed on a monthly basis. One conventional SET cycle might be replaced by three natural or mildly stimulated IVF/ICSI cycles, considering the higher cancellation rate and lower pregnancy rate.

Finally, it is important to realize that the exclusion of the first cycle for reimbursement has to change. All three cycles should be reimbursed. The high initial costs are too high for lower-class couples and thus lead to unequal access to reproductive care. Moreover, the high costs of the first

cycle will stop the trend to SET and thus will lead to undesirable high rates of multiples.

Future research

Two cyles with single embryo transfer seems equally effective as one cycle with double embryo transfer in an IVF population prone to twin pregnancies and the medical costs per live birth up to six weeks after delivery are the same. More than Euro 7,000 per live birth will be saved after implementing SET, if lifetime costs for severe handicaps are included. To make SET even more cost-effective and therefore better accepted by the patient, as well as the fertility specialist and the government, the live birth rate has to be improved. Fundamental research can contribute to more knowledge about the implantation process of the embryo into the decidua, which remains a difficult step in the achievement of a pregnancy after IVF (part II of this thesis). Another way to improve the pregnancy rate is to enhance the results of cryopreservation programmes to increase the cumulative live birth rate per started cycle. Since only one embryo will be transferred after SET more embryos are available for this procedure. The costs of the cryopreservation procedure and the transfer of frozen embryos is less than one stimulated IVF cycle. A third manner to improve the pregnancy rate after IVF is the search for better parameters for embryo quality that are highly correlated to subsequent pregnancy. We could, for example, focus on substances in follicular fluid like immune cells (**chapter 5**). Pre-implantation Genetic Screening is also a promising technique to select genetically normal embryos with a high implantation potential.

It would also be interesting to investigate the efficiency of SET in a broader group of IVF patients, less prone to twin pregnancies. How efficient is SET in a third or fourth IVF cycle, in older women, with low quality embryos?

Furthermore, we have to gain more insight in the preference of infertility patients to SET or DET in order to facilitate the implementation of SET by a better acceptance in this group of patients. Murray and colleagues showed already that additional information about the risks of twins did not change couples' attitude towards SET, but maintaining existing pregnancy rates and offering a fixed charge for all embryo trans-

fers resulting from one started cycle may encourage more couples to consider SET (32).

Finally, what will be the hindering factors when SET would be implemented in daily practice and what are the best strategies to overcome these obstacles. As mentioned before, the first obstacle could be the desire for a twin over a singleton pregnancy by infertile couples. A second major obstacle for both patients and fertility specialists is thought to be the restricted reimbursement of only the second and third IVF/ICSI treatment cycle. Both may prefer cycles with DET instead of SET, because of an increased total chance for pregnancy. Implementation of SET cannot be overcome by legislation adaptation only. Olofsson et al. just recently showed that in Sweden after a legislation update that states SET fundamentally, the SET rate got stuck on 61% (33).

Part II: Immunological Aspects

NK cells and pregnancy

We were interested in reducing the multiple pregnancy rate after IVF, without decreasing the pregnancy rate as such. As shown in this thesis single embryo transfer can decrease the number of multiple pregnancies substantially, with only a slight drop in pregnancy rate. To make implementation of single embryo transfer not only better accepted, but also applicable to a broader group of patients, the overall pregnancy rate of an IVF clinic has to be high. In our view pregnancy rates can be increased if the implantation of the embryo into the decidua takes place under optimal conditions. So, we set out to gain more insight into the immunological interaction between the fetus and the mother at the time of implantation. We focused on natural killer (NK) cells, because they play an important role in human implantation. A characteristic feature of human endometrium and decidua during reproductive life is the presence of a large population of natural killer cells. In pregnancy up to 70% of decidual leukocytes are NK cells. They accumulate at the implantation site where they are found in close proximity to invading trophoblast. After about the twentieth week of gestation, trophoblast invasion is complete, and at this time the number of uterine NK cells is thought to decline.

Large numbers of NK cells are not a feature of other solid tissues. Another remarkable feature of uterine NK cells is the expression of specific NK cell receptors on their surface which interact with HLA molecules expressed on the invading trophoblast cells (HLA-CW, HLA-E and HLA-G). It is suggested that interaction between HLA-G (fetal) and uterine NK cells (maternal) induces a combination of positive and negative signals that regulate cytokine production, proliferation and cytolysis in order to make successful placentation possible (34-37). Thus, it is imperative to gain more insight in the precise role of uNK cells in implantation in normal as well as pathologic pregnancies. It is well possible that NK cells also play a role in reproductive processes during stages preceding implantation, for example in oocyte maturation. Both follicular fluid and uterine NK cells might be valuable parameters for the quality of oocytes and/or endometrium.

In IVF treatment, hormonal stimulation of the ovaries is used to induce multiple follicular growth. This ovarian hyperstimulation results in supraphysiological concentrations of sex steroids (estradiol and progesterone) that might have a deleterious effect on endometrial receptivity. In fact, both positive and negative effects of ovarian hyperstimulation on pregnancy rates after IVF have been reported (38-41). Hormones do have an effect on the number and possibly the function of leukocytes, as the number of leukocytes fluctuate substantially during the menstrual cycle and throughout pregnancy. Moreover, estrogen receptors have been detected on NK cells (42), although this was not confirmed by Stewart et al. (43).

We investigated the repercussion of hormonal hyperstimulation on the NK cell composition of endometrium from IVF patients compared with the endometrium of naturally cycling women during the window of implantation (**chapter 6**). We found that hormonal stimulation positively affected the $CD56^{bright}/CD56^{dim}$ ratio of the endometrium caused by a relative decrease in the cytotoxic $CD56^{dim}CD16^{+}$ NK cell number. This NK cell subset has mainly cytotoxic properties and is thought to be detrimental for implantation, whereas the $CD56^{bright}CD16^{-}$ NK cell population is associated with successful implantation and placental maturation (34). So, we suggest that supraphysiological levels of sex steroids after IVF treatment do not impair endometrium receptivity, indeed it might even be beneficial. This idea is endorsed by the fact that histologically no endometrial advancement was shown in the IVF group.

We discovered a third subset of NK cells in the endometrium at the time that it is receptive for implantation, which has not been described previously. We showed that, based upon the NK cell marker CD56, the $CD56^{bright}$ population could be subdivided into two different populations. The so-called $CD56^{superbright}$ cells, which are CD16 negative, are unique in the sense that they were detected only in the endometrium and not in peripheral blood. Based upon this finding, we defined three distinct NK cell populations in the uterus, i.e. $CD56^{dim}$, $CD56^{bright}$ and $CD56^{superbright}$. Furthermore, in contrast to both uterine and peripheral $CD56^{bright}$ NK cells, a large proportion of $CD56^{superbright}$ NK cells expressed NK cell receptors for HLA-Cw and -G molecules. Practically all $CD56^{superbright}$ expressed NKG2A at high levels. This latter receptor is reported to bind HLA-E. These features of $CD56^{superbright}$ NK cells suggest different functional properties as compared with $CD56^{bright}$ cells. Maybe it is this $CD56^{superbright}$ subset of the $CD56^{bright}$ NK cells that is responsible for the favorable effects on reproduction as described above.

The results of this study might be a first step for further investigations to use the number and function of NK cell subsets as a parameter for endometrium quality by linking these data to subsequent outcome of pregnancy. This may support improvement of stimulation protocols in assisted reproductive technology.

Next to the implantation phase, NK cells may also be of importance in the very early stages of reproduction. It is known that leukocytes are present in follicular fluid (FF). NK cells in FF might well be involved in oocyte development and maturation. We hypothesized that follicular fluid provides the environment in which oocyte maturation occurs and therefore may influence the quality of the oocyte. The composition of the fluid may thus be linked to fertilization and early embryonic development and disturbed cellular immunity in follicular fluid might be the cause of aberrant oocyte development.

Aberrant oocyte development can provide a possible explanation for the 'idiopathic' cause of infertility, because the fertilization rate of these oocytes is lower as compared to oocytes from patients suffering from tubal factor infertility (44-46). To investigate this hypothesis we determined the levels of NK and T cell subsets by flowcytometric analysis in separately collected follicular fluids of patients with idiopathic infertility and compared these levels to FF of a control group consisting of couples with severe male and tubal factor infertility (**chapter 5**). We found that

the proportion of FF $CD56^{dim}CD16^{+}$NK cells in women suffering from idiopathic infertility was significantly higher than in controls. The excess of $CD56^{dim}CD16^{+}$NK cells in follicular fluid could reveal a cause for oocyte 'dysfunction' and therefore for the idiopathic infertility in this group, because the $CD56^{dim}CD16^{+}$NK cell subset is generally considered to be cytotoxic and is negatively associated with reproductive events.

It would be interesting to correlate NK cell subset levels and function in FF to subsequent quality of embryos and their implantation capacity. Monofollicular growth in case of natural cycle IVF and ICSI offers an excellent opportunity to study the correlation between cellular immune cells in FF and pregnancy rate, although the influence of hormonal stimulation cannot be measured. When data is gathered on a large scale, subgroup analysis can be performed to investigate for instance the influence of smoking or the use of painkillers on leukocyte composition of the follicles.

An intriguing question is where the FF leukocytes come from. Do they originate from a distinct population or are they recruited from the peripheral blood circulation and undergo tissue-specific differentiation? Piccinni and co-workers (47) reported a qualitative, tissue-specific difference in functionality between lymphocytes present in the cumulus cells of a follicle as compared to peripheral blood lymphocytes. These lymphocytes produce a different profile and amount of cytokines. It will be interesting to determine cytokines and chemokines in FF that could attract NK cells to the follicle. This will enable us to investigate NK cell traffic and possibly influence these processes to enhance oocyte quality and subsequent pregnancy rate after IVF.

Function of uterine NK cells

NK cells appear instrumental in successful decidualization. Their function is modulated through interaction with HLA-molecules expressed on invading trophoblast cells. We paid particular attention to HLA-G, because HLA-G has a restricted tissue distribution and low polymorphism. In the placenta, HLA-G is expressed by the extra-villous (invasive) trophoblast cells at the fetal-maternal interface. Also, it is found to be present in amniotic fluid. Initially, it was thought that HLA-G had a strictly inhibitory function in order to protect the semi-allogenic fetus

from an attack from the maternal immune system. Nowadays, it becomes clear that the most likely function of HLA-G is to induce the production of cytokines and angiogenic factors by uterine NK cells to alter trophoblast invasion and differentiation in order to supply an appropriate blood flow to the placenta (48). This would suggest a more finely tuned modulatory function for HLA-G rather than a strictly inhibitory role.

In **chapter 7** we investigated the effect of membrane-bound HLA-G (mHLA-G) on production of various cytokines related to implantation, i.e IFN-γ, Vascular Endothelial Growth Factor (VEGF), Interleukin 3 (IL3) and Leukaemia Inhibitory Factor (LIF) (49-52). We also investigated mHLA-G induced proliferation of the uterine mononuclear cell (UMC) population as a whole, and uterine NK cells in particular from endometrium of non-pregnant woman during the phase that the endometrium is receptive for implantation.

We have shown that mHLA-G stimulated specific functions of uNK cells, like proliferation and cytokine production. Notably, the same functions were suppressed in T cells. Studies on uterine lymphocytes so far only pointed towards an inhibitory role for mHLA-G in protection of the semiallogeneic foetus by maternal NK and T cells. This was achieved by inhibition of NK cell cytotoxicity (53) and downmodulation of Th1 type cytokine production (36;37). Upregulation of the Th2 response by mHLA-G was found in peripheral lymphocytes, but was not observed for uterine lymphocytes (36). Our results indicate that a delicate balance exists between active processes to promote proper implantation, while at the same time inhibitory processes must protect the fetus from a generalized attack by the maternal immune system.

In our study the mononuclear cells were derived from non-pregnant endometrium at the time that it is receptive for implantation. These endometrial cells have not yet been in contact with fetal trophoblast cells and/or have not yet been affected by the altered hormone and cytokine levels that occur during pregnancy. Therefore this tissue is most appropriate to study the interaction of NK cells with HLA-G, because it has been shown that decidual NK cells express different activation markers and adhesion molecules as compared to those from non-pregnant endometrium (54). It is well conceivable that also the functional capacities of cells derived from non-pregnant endometrium differ from decidual tissue from pregnant women. Most other research groups, however,

used decidual tissue from elective abortions to study the interaction between HLA-G and uterine cells (34;36;37;53).

In conclusion, our data suggest an active role for mHLA-G in modulating uterine NK cells that are present in the endometrium at the time that it is receptive for implantation. This indicates that mHLA-G is instrumental in active fine-tuning of the local immune response in order to promote successful implantation. Besides HLA-G, two other HLA-molecules are present on trophoblast cells, namely HLA-E and HLA-CW. HLA-E, like HLA-G, is also a non-classical class I molecule with limited polymorphism. Previous studies using HLA-G transfectants are hampered by the co-expression of HLA-E. The cell-surface expression of HLA-E is dependent on its binding of signal peptides cleaved from other class I molecules, such as HLA-G. Signal nonamer peptides from both HLA-G and HLA-CW have been shown to bind to HLA-E (55-57). The receptor CD94/NKG2A recognizes HLA-E on extra-villous trophoblast. It can well be assumed that HLA-E has a different function as compared to HLA-G, notably its receptor on NK cells shows a different expression pattern than HLA-G receptors. A significantly higher number of $CD56^{superbright}$ NK cells as compared to $CD56^{bright}$ NK cells express CD94/NKG2A and also the level of expression is higher in this group as compared to $CD56^{bright}$ NK cells (**chapter 6**). It is most likely that the combination of HLA-G and HLA-E loaded with HLA-G derived nonamer peptide, provides the unique signals for modulating local uterine immune responses to facilitate implantation.

Clinical Implications

Immunotherapy for pregnancy failure

Recent reports in the media and on the internet have exposed women to a baffling array of conflicting information about tests for NK cells and "cures" for infertility and miscarriage. These authors assume that malfunction of NK cells causes pregnancy failure and are offering blood tests to measure the number and activity of peripheral blood NK cells. As a consequence of these test results these women are exposed to immunotherapy such as steroids, intravenous immunoglobulins, paternal or donor cell immunization and cytokine blocking agents (58). This is a dangerous development, because there is not enough scientific know-

ledge yet of the specific underlying immunological pathophysiology of pregnancy failure. Therefore, immunotherapy could have its effect the wrong way. Moreover, there is no scientific evidence of the beneficial effect of these therapies on pregnancy failure. Up to now, most of these tests aim at the number and activity of peripheral blood leukocytes, especially the NK cells. It is becoming more clearly now that the uterine NK cells play a crucial role in implantation and that these cells are phenotypically and functionally different from peripheral NK cells and should be regarded as a separate lyphoid subset. Evidence is accumulating that the function of uterine NK cells must nót be inhibited but exactly activated. Uterine NK cells probably have an essential, beneficial effect on trophoblast by secreting cytokines that alter the depth of placental invasion. Therefore, current immunotherapies that suppress NK cell activity in a general way could have a detrimental effect on pregnancy.

Many clinical trials that investigate the effects of immunotherapy on pregnancy outcome show an inadequate sample size, poor definition of heterogeneity and lack proper stratification to minimize the effects of heterogeneity. Another weak point is that most trials also lack verification of the expected alteration in immunophysiology in the recipients (59). Daya and colleagues performed a meta-analysis to determine whether intravenous immunoglobulin (IVIG) improved the chances of successful pregnancy in women with recurrent miscarriage by using individual patient data from efficacy trials. They found no significant improvement in successful pregnancy or live birth rate with IVIG (60). Furthermore, a systemic Cochrane review also states that various immunological treatments in women with recurrent miscarriage provide no significant beneficial effect over placebo in preventing further miscarriages (61).

In reproductive medicine it is very important to evaluate treatments carefully before implementation in daily practice. Theoretically, it might be possible to intervene with selection of nature and maintain pregnancies with for instance chromosome abnormalities.

In our opinion, it remains of utmost importance to perform extensive fundamental research to understand the process of implantation, before immunotherapies will be applied to infertility patients. Today, it is too early and risky to perform immunotherapy in daily IVF practice.

Future research

HLA-CW is the third HLA molecule present on fetal trophoblast cells. HLA-CW, in contrast to HLA-G and HLA-E, is a polymorphic classical MHC class I molecule. The paternal allele is expressed on the extra-villous trophoblast cell surface and may be important for allorecognition of trophoblast. HLA-CW shows ubiquitous expression in the human body. Up to now, little is known about the precise role of HLA-CW in implantation. Hiby et al. have proposed a new theory about the role of HLA-CW in the pathogenesis of preeclampsia (62).

Preeclampsia is a serious disorder of human pregnancy in which the placenta receives an inadequate supply of blood due to failure of trophoblast invasion. The maternal systemic illness is thought to be a late manifestation of the placental ischaemia that is secondary to the reduced utero-placental blood flow (63). In genetic studies as well as in population-based studies a contribution of both paternal and maternal factors and genotype is apparent (64). The increased risk of preeclampsia in first pregnancies and after changing partners suggests that immune recognition is involved (65). Strikingly, there is also a greatly increased risk in women who have received donated oocytes in an IVF program (66). In this case the fetus will share neither maternal nor paternal genes.

Hiby and colleagues implicate that HLA-CW on fetal trophoblast and Killer cell Immunoglobulin-like Receptors (KIR) on uterine NK cells play an important immunological role in preeclampsia. HLA-CW, as trophoblast ligand, is known to have extensive polymorphism in contrast to HLA-G and HLA-E. On the other hand, the KIR family of NK cell receptors is also polymorphic. Therefore HLA-CW and KIR are important for allorecognition and are probably relevant for the development of preeclampsia. KIR haplotypes can be divided into two groups. The A haplotypes are mainly characterized by the presence of inhibitory receptors, while the B haplotypes presents mainly activating receptors. The HLA-CW allotypes can be grouped into HLAI (weak inhibition upon interaction with KIR) and HLA-C2 (strong inhibition upon interaction with KIR). Hiby et al. found preeclampsia to be more prevalent in women who are homozygous for A KIR haplotype (AA) than women who are either heterozygous (AB) or homozygous for group B haplotypes (BB). They also found that the increased prevalence of preeclampsia in AA KIR women was entirely due to pregnancies where the fetus

genotype was either homozygous C2 or heterozygous C1C2. So, it is hypothesized that an absence of activating KIR favors preeclampsia, because too much inhibition of uNK cells will lead to poor trophoblast invasion into the uterine arteries. These results indicate a possibly important role for fetal HLA-CW on trophoblast cells in implantation through interaction with maternal KIR on uNK cells.

In the light of these results it is difficult to explain why a first pregnancy protects against the development of preeclampsia or at least causes a milder form of the disease in subsequent pregnancies with the same partner. Although NK cells do not exhibit memory, it is speculated that in subsequent pregnancies with the same AA-C2 combination a weaker inhibition would occur via the NK receptor KIR2DL1. This can be achieved by a reduced expression level of KIR2DL1 on NK cells in subsequent pregnancies or by a decreased presence of KIR2DL1 positive cells at the implantation site.

Once more the regulation of the development of the placenta seems an active process, rather than suppression of the maternal immune system against her semi-allogeneic fetus.

Furthermore, we would like to draw attention to regulatory T cells. Evidence is accumulating that, next to NK cells, regulatory T cells in the decidua and peripheral blood may also play an important role in protecting the fetus from alloreactive immune responses against paternal antigens at the maternal-fetal interface. Immunosuppressive regulatory T cells (Treg) are already known to be crucial in the induction and maintenance of transplantation tolerance (67;68). Regulatory T cells are a specialized population of T cells that suppress T cell responses. The absolute number of circulating maternal Treg cells and decidual Treg cells, defined as $CD4^+CD25^+$ cells, increases progressively at each stage in human pregnancy starting from the first trimester (69-71). Increased numbers of systemic Treg cells and altered function might explain why some autoimmune syndromes, such as rheumatoid arthritis, go into remission during pregnancy (72).

Sasaki et al. demonstrated that the proportion of decidual Treg cells was significantly lower in samples from patients suffering from spontaneous abortions (71). In murine pregnancy it has been shown that transfer of maternal T cells depleted of Treg cells resulted in uniform failure of allogeneic pregnancies, whereas this manupilation did not affect the outcome of matings involving genetically identical male and female mice

(syngeneic pregnancies). The ability to suppress maternal immunity to fetal alloantigens was dependent on expansion of the maternal Treg pool. This response was not driven by fetal alloantigens, because expansion also occurred in females mated with syngeneic males (73). Hormonal changes during pregnancy might provide an explanation for enhanced maternal Treg development during gestation as was shown by Polanczyk and colleagues. They demonstrated that estradiol treatment induces a similar increase in number and function of Treg cells as does pregnancy. Expansion of Treg number and function during pregnancy may be responsible not only for preventing rejection of the fetus, but also for protecting the mother from developing (relapses) of autoimmunne diseases (74).

Regulatory T cells may play an important role in reproduction, especially during implantation. It will be interesting to investigate whether these cells interact with NK cell function. Do Treg cells influence the process of implantation directly or indirectly possibly via NK cells? Do they express specific receptors for HLA-G, HLA-E and HLA-CW on trophoblast cells?

Implantation and development of the human fetus is for the greater part a mystery. In the field of local immunity at the implantation site much remains to discover. In this thesis the role of uterine NK cells and HLA-G in implantation was further explored. Future research on the interaction of NK cells and HLA-G with other immune cells and processes, like Treg cells and the complex of HLA-CW with uterine NK cell receptors, might contribute to unravel the mystery of human implantation. When we gain more insight into the immunological processes of implantation it might be possible to increase the pregnancy rate, while reducing the multiple pregnancy rate in IVF by implementing single embryo transfer.

Appendix: calculation of costs

VARIABLES:

n	= number of IVF treatment cycles (n = 789)
C_{tot}	= costs IVF-cycle + costs IVF-pregnancy up to 6 weeks post-partum + lifetime costs handicapped children
$C_{IVF\text{-}cycle}$	= costs IVF-cycle (2,532 euro)
$C_{preg\text{-}twin}$	= costs IVF twin pregnancy up to 6 weeks post partum (13,469 euro)
$C_{preg\text{-}sing}$	= costs IVF singleton pregnancy up to 6 weeks post partum (2,550 euro)
$C_{hand\text{-}twin}$	= lifetime costs of handicapped twin per twin birth (20,477 euro)
$C_{hand\text{-}sing}$	= lifetime costs of handicapped singleton per singleton birth (1,489 euro)
P_{preg}	= overall pregnancy rate (before implementing SET) (23%)
P_{twin}	= overall twin pregnancy rate (before implementing SET) (27%)
P_{SET}	= chance to be included for SET (41%)
P_{DET}	= chance to be included for DET (1- PSET) (59%)
$P_{preg\text{-}SET}$	= pregnancy rate after SET (first cycle) (26%)
$P_{preg\text{-}DET}$	= pregnancy rate after DET (14%)
$P_{twin\text{-}DET}$	= twin pregnancy rate after DET (12.5%)

Appendix a:

Before implementation of SET (Nijmegen):

$$C_{tot} = n \times C_{IVF\text{-}cycle} + n \times P_{preg} \times P_{twin} \times (C_{preg\text{-}twin} + C_{hand\text{-}twin}) + n \times P_{preg} \times (1 - P_{twin}) \times (C_{preg\text{-}sing} + C_{hand\text{-}sing})$$

After implementation of SET (Nijmegen):

$$C_{tot} = n \times C_{IVF\text{-}cycle} + n \times P_{SET} \times P_{preg\text{-}SET} \times (C_{preg\text{-}sing} + C_{hand\text{-}sing}) + n \times P_{DET} \times P_{preg\text{-}DET} \times P_{twin\text{-}DET} \times (C_{preg\text{-}twin} + C_{hand\text{-}twin}) + n \times P_{DET} \times P_{preg\text{-}DET} \times (1\text{-}P_{twin\text{-}DET}) \times (C_{preg\text{-}sing} + C_{hand\text{-}sing})$$

Appendix b:

In case 1.5 more SET cycles will be performed to equalize the pregnancy rate to DET in the same population. The costs of IVF cycles will become 1.2 times higher; $1.5 \times n \times P^{SET} + n \times P^{DET}$. The costs for the SET group will become 1.5 times higher than for the DET group.

$$C_{tot} = \mathbf{1.2} \times n \times C_{IVF\text{-}cycle} + \mathbf{1.5} \times n \times P_{SET} \times P_{preg\text{-}SET} \times (C_{preg\text{-}sing} + C_{hand\text{-}sing}) + n \times P_{DET} \times P_{preg\text{-}DET} \times P_{twin\text{-}DET} \times (C_{preg\text{-}twin} + C_{hand\text{-}twin}) + n \times P_{DET} \times P_{preg\text{-}DET} \times (1\text{-}P_{twin\text{-}DET}) \times (C_{preg\text{-}sing} + C_{hand\text{-}sing})$$

note: In the DET group the costs of IVF pregnancy up to six weeks after delivery together with the lifetime costs for handicapped children are measured for both singleton and twin pregnancies.

References

1. Bergh T, Ericson A, Hillensjo T, Nygren KG, Wennerholm UB. Deliveries and children born after in-vitro fertilisation in Sweden 1982-95: a retrospective cohort study. Lancet 1999;354(9190):1579-85.
2. Koudstaal J, Braat DD, Bruinse HW, Naaktgeboren N, Vermeiden JP, Visser GH. Obstetric outcome of singleton pregnancies after IVF: a matched control study in four Dutch university hospitals. Hum.Reprod. 2000;15(8):1819-25.
3. Koudstaal J, Bruinse HW, Helmerhorst FM, Vermeiden JP, Willemsen WN, Visser GH. Obstetric outcome of twin pregnancies after in-vitro fertilization: a matched control study in four Dutch university hospitals. Hum.Reprod. 2000;15(4):935-40.
4. Koivurova S, Hartikainen AL, Gissler M, Hemminki E, Sovio U, Jarvelin MR. Neonatal outcome and congenital malformations in children born after in-vitro fertilization. Hum.Reprod. 2002;17(5):1391-8.
5. Klemetti R, Gissler M, Hemminki E. Comparison of perinatal health of children born from IVF in Finland in the early and late 1990s. Hum.Reprod. 2002;17(8):2192-8.
6. Helmerhorst FM, Perquin DA, Donker D, Keirse MJ. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. BMJ 2004;328(7434):261.
7. Jackson RA, Gibson KA, Wu YW, Croughan MS. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. Obstet.Gynecol. 2004;103(3):551-63. Adang EMM. Debatable Efficiency. Economische Statistische Berichten 2002;4349:170-171.
9. Gerris J, De Sutter P, De Neubourg D, Van Royen E, Vander Elst J, Mangelschots K et al. A real-life prospective health economic study of elective single embryo transfer versus two-embryo transfer in first IVF/ICSI cycles. Hum.Reprod. 2004;19(4):917-23.
10. Wolner-Hanssen P, Rydhstroem H. Cost-effectiveness analysis of in-vitro fertilization: estimated costs per successful pregnancy after transfer of one or two embryos. Hum.Reprod. 1998;13(1):88-94.
11. De Sutter P, Gerris J, Dhont M. A health-economic decision-analytic model comparing double with single embryo transfer in IVF/ICSI. Hum.Reprod. 2002;17(11):2891-6.
12. Bergh C. Single embryo transfer: a mini-review. Hum.Reprod. 2005;20(2):323-7.

13. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Vercruyssen M, Barudy-Vasquez J et al. Elective single day 3 embryo transfer halves the twinning rate without decrease in the ongoing pregnancy rate of an IVF/ICSI programme. Hum.Reprod. 2002;17(10):2626-31.
14. Tiitinen A, Unkila-Kallio L, Halttunen M, Hyden-Granskog C. Impact of elective single embryo transfer on the twin pregnancy rate. Hum.Reprod. 2003;18(7):1449-53.
15. Vilska S, Tiitinen A, Hyden Granskog C, Hovatta O. Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. Hum.Reprod. 1999;14(9):2392-5.
16. De Sutter P, Van der EJ, Coetsier T, Dhont M. Single embryo transfer and multiple pregnancy rate reduction in IVF/ICSI: a 5-year appraisal. Reprod. Biomed.Online. 2003;6(4):464-9.
17. Catt J, Wood T, Henman M, Jansen R. Single embryo transfer in IVF to prevent multiple pregnancies. Twin.Res. 2003;6(6):536-9.
18. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de MM, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. Hum.Reprod. 1999;14(10):2581-7.
19. Martikainen H, Tiitinen A, Tomas C, Tapanainen J, Orava M, Tuomivaara L et al. One versus two embryo transfer after IVF and ICSI: a randomized study. Hum.Reprod. 2001;16(9):1900-3.
20. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: a prospective randomized trial. Fertil.Steril. 2004;81(3):551-5.
21. Thurin A, Hausken J, Hillensjo T, Jablonowska B, Pinborg A, Strandell A et al. Elective single-embryo transfer versus double-embryo transfer in in vitro fertilization. N.Engl.J.Med. 2004;351(23):2392-402.
22. van Montfoort AP, Dumoulin JC, Land JA, Coonen E, Derhaag JG, Evers JL. Elective single embryo transfer (eSET) policy in the first three IVF/ICSI treatment cycles. Hum.Reprod. 2005;20(2):433-6.
23. Nygren KG, Andersen AN. Assisted reproductive technology in Europe, 1999. Results generated from European registers by ESHRE. Hum.Reprod. 2002;17(12):3260-74.
24. Tiitinen A, Halttunen M, Harkki P, Vuoristo P, Hyden-Granskog C. Elective single embryo transfer: the value of cryopreservation. Hum.Reprod. 2001;16(6):1140-4.

25. Pelinck MJ, Hoek A, Simons AHM, Heineman MJ. Efficacy of natural cycle IVF: a review of the literature. Hum.Reprod. Update 2002;8(2):129-39.
26. Pelinck MJ, Vogel NE, Hoek A, Arts EG, Simons AH, Heineman MJ. Minimal stimulation IVF with late follicular phase administration of the GnRH antagonist cetrorelix and concomitant substitution with recombinant FSH: a pilot study. Hum.Reprod. 2005;20(3):642-8.
27. Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. J.Clin.Endocrinol.Metab 2003;88(1):166-73.
28. Hojgaard A, Ingerslev HJ, Dinesen J. Friendly IVF: patient opinions. Hum.Reprod. 2001;16(7):1391-6.
29. Frankfurter D, Barett C, Oskowitz S, Alper M, Berger M, and Penzias A. Insurance mandates for IVF coverage effectively lower multiple births per embryo transfer. Fertil.Steril. 70(1 Suppl), 51S. 1998.
30. Tarun J, Harlow B, and Hornstein M. Insurance coverage and outcomes of in vitro fertilization. N.Engl.J.Med. 347, 661-666. 2002.
31. Reynolds MA, Schieve LA, Jeng G, Peterson HB. Does insurance coverage decrease the risk for multiple births associated with assisted reproductive technology? Fertil.Steril. 2003;80(1):16-23.
32. Murray S, Shetty A, Rattray A, Taylor V, Bhattacharya S. A randomized comparison of alternative methods of information provision on the acceptability of elective single embryo transfer. Hum.Reprod. 2004;19(4):911-6.
33. Olofsson, J. I. Effects of novel legislation on embryo transfer policy, results and pregnancy outcome in a Swedish IVF unit. Human Reproduction 19(Suppl 1), i59-i60. 2004.
34. King A, Allan DS, Bowen M, Powis SJ, Joseph S, Verma S et al. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. Eur.J.Immunol. 2000;30(6):1623-31.
35. Hunt JS, Petroff MG, Burnett TG. Uterine leukocytes: key players in pregnancy. Semin.Cell Dev.Biol. 2000;11(2):127-37.
36. Kanai T, Fujii T, Unno N, Yamashita T, Hyodo H, Miki A et al. Human leukocyte antigen-G-expressing cells differently modulate the release of cytokines from mononuclear cells present in the decidua versus peripheral blood. Am.J.Reprod.Immunol. 2001;45(2):94-9.

37. Rieger L, Hofmeister V, Probe C, Dietl J, Weiss EH, Steck T et al. Th1- and Th2-like cytokine production by first trimester decidual large granular lymphocytes is influenced by HLA-G and HLA-E. Mol.Hum.Reprod. 2002;8(3):255-61.
38. Paulson RJ, Sauer MV, Lobo RA. Embryo implantation after human in vitro fertilization: importance of endometrial receptivity. Fertil.Steril. 1990;53(5):870-4.
39. Chenette PE, Sauer MV, Paulson RJ. Very high serum estradiol levels are not detrimental to clinical outcome of in vitro fertilization. Fertil.Steril. 1990;54(5):858-63.
40. Simon C, Cano F, Valbuena D, Remohi J, Pellicer A. Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. Hum.Reprod. 1995;10(9):2432-7.
41. Macklon NS, Fauser BC. Impact of ovarian hyperstimulation on the luteal phase. J.Reprod.Fertil.Suppl 2000;55:101-8.
42. Henderson TA, Saunders PT, Moffett-King A, Groome NP, Critchley HO. Steroid receptor expression in uterine natural killer cells. J.Clin.Endocrinol.Metab 2003;88(1):440-9.
43. Stewart JA, Bulmer JN, Murdoch AP. Endometrial leucocytes: expression of steroid hormone receptors. J.Clin.Pathol. 1998;51(2):121-6.
44. Mahadevan MM, Trounson AO, Leeton JF. The relationship of tubal blockage, infertility of unknown cause, suspected male infertility, and endometriosis to success of in vitro fertilization and embryo transfer. Fertil.Steril. 1983;40(6):755-62.
45. Audibert F, Hedon B, Arnal F, Humeau C, Badoc E, Virenque V et al. Results of IVF attempts in patients with unexplained infertility. Hum.Reprod. 1989;4(7):766-71.
46. Lipitz S, Rabinovici J, Ben Shlomo I, Bider D, Ben Rafael Z, Mashiach S et al. Complete failure of fertilization in couples with unexplained infertility: implications for subsequent in vitro fertilization cycles. Fertil.Steril. 1993;59(2):348-52.
47. Piccinni MP, Scaletti C, Mavilia C, Lazzeri E, Romagnani P, Natali I et al. Production of IL-4 and leukemia inhibitory factor by T cells of the cumulus oophorus: a favorable microenvironment for pre-implantation embryo development. Eur.J.Immunol. 2001;31(8):2431-7.
48. Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K et al.

Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. J.Clin.Endocrinol.Metab 2001;86(4):1823-34.
49. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 1992;359(6390):76-9.
50. Abulafia O, Sherer DM. Angiogenesis of the endometrium. Obstet.Gynecol. 1999;94(1):148-53.
51. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. J.Exp.Med. 2000;192(2):259-69.
52. Di Simone N, Caliandro D, Castellani R, Ferrazzani S, Caruso A. Interleukin-3 and human trophoblast: in vitro explanations for the effect of interleukin in patients with antiphospholipid antibody syndrome. Fertil. Steril. 2000;73(6):1194-200.
53. Rouas Freiss N, Goncalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc.Natl.Acad.Sci.U.S.A. 1997;94(21):11520-5.
54. Kodama T, Hara T, Okamoto E, Kusunoki Y, Ohama K. Characteristic changes of large granular lymphocytes that strongly express CD56 in endometrium during the menstrual cycle and early pregnancy. Hum.Reprod. 1998;13(4):1036-43.
55. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA- E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. Eur.J.Immunol. 1997;27(5):1164-9.
56. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)- E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J.Exp.Med. 1998;187(5):813-8.
57. Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. J.Immunol. 1998;160(10):4951-60.
58. Moffett A, Regan L, Braude P. Natural killer cells, miscarriage, and infertility. BMJ 2004;329(7477):1283-5.

59. Clark DA, Coulam CB, Daya S, Chaouat G. Unexplained sporadic and recurrent miscarrage in the new millennium: a critical analysis of immune mechanisms and treatments. Hum.Reprod.Update. 2001;7(5):501-11.
60. Daya S, Gunby J, Clark DA. Intravenous immunoglobulin therapy for recurrent spontaneous abortion: a meta-analysis. Am.J.Reprod.Immunol. 1998;39(2):69-76.
61. Scott JR. Immunotherapy for recurrent miscarriage. Cochrane.Database.Syst.Rev. 2003(1):CD000112.
62. Hiby SE, Walker JJ, O'shaughnessy KM, Redman CW, Carrington M, Trowsdale J et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. J.Exp.Med. 2004;200(8):957-65.
63. Redman CW. Current topic: pre-eclampsia and the placenta. Placenta 1991;12(4):301-8.
64. Esplin MS, Fausett MB, Fraser A, Kerber R, Mineau G, Carrillo J et al. Paternal and maternal components of the predisposition to preeclampsia. N.Engl.J.Med. 2001;344(12):867-72.
65. Trupin LS, Simon LP, Eskenazi B. Change in paternity: a risk factor for preeclampsia in multiparas. Epidemiology 1996;7(3):240-4.
66. Soderstrom-Anttila V, Tiitinen A, Foudila T, Hovatta O. Obstetric and perinatal outcome after oocyte donation: comparison with in-vitro fertilization pregnancies. Hum.Reprod. 1998;13(2):483-90.
67. Kilshaw PJ, Brent L, Pinto M. Suppressor T cells in mice made unresponsive to skin allografts. Nature 1975;255(5508):489-91.
68. Dorsch S, Roser B. T cells mediate transplantation tolerance. Nature 1975;258(5532):233-5.
69. Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. Immunology 2004;112(1):38-43.
70. Heikkinen J, Mottonen M, Alanen A, Lassila O. Phenotypic characterization of regulatory T cells in the human decidua. Clin.Exp.Immunol. 2004;136(2):373-8.
71. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol.Hum.Reprod. 2004;10(5):347-53.
72. Ostensen M, Villiger PM. Immunology of pregnancy-pregnancy as a

remission inducing agent in rheumatoid arthritis. Transpl.Immunol. 2002;9(2-4):155-60.
73. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat.Immunol. 2004;5(3):266-71.
74. Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenbark AA, Ziegler SF et al. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. J.Immunol. 2004;173(4):2227-30.

Samenvatting

Het optreden van meerlingzwangerschappen is de meest ernstige complicatie van IVF behandelingen. De incidentie van maternale en perinatale morbiditeit en mortaliteit is significant hoger dan van éénlingzwangerschappen. Daarom hebben de patiënten die zwanger zijn van een meerling en de meerlingen zelf meer medische zorg nodig. In **hoofdstuk 1** wordt in deel I achtergrond informatie gegeven over de medische, sociale en economische problemen die gerelateerd zijn aan meerlingzwangerschappen na IVF. Tevens wordt een aantal behandelingsstrategieën beschreven welke het aantal meerlingen na IVF verminderen: het terugplaatsen van één embryo (single embryo transfer (SET)), IVF in de natuurlijke cyclus en selectieve embryo reductie. SET lijkt een goede optie om een daling van het aantal meerlingen te bewerkstelligen, echter het zwangerschapspercentage zal naar verwachting door deze strategie ook enigszins dalen. Om de acceptatie van de implementatie van SET te verhogen is het daarom noodzakelijk om de zwangerschapskans na IVF te verbeteren, met als doel de zwangerschapskans na SET op hetzelfde niveau te brengen als de kans na transfer van twee embryo's (double embryo transfer (DET)). De implantatie van een embryo is een cruciale stap in de totstandkoming van een succesvolle zwangerschap. Om de zwangerschapskans na IVF te verbeteren is het daarom belangrijk om meer fundamenteel onderzoek te verrichten naar processen die een rol spelen bij de implantatie. In dit kader is het interessant om immunologische processen die tijdens de implantatie plaatsvinden te bestuderen. Niet alleen is de foetus een semi-allograft die geaccepteerd moet worden door het maternale immuun systeem (50% van de genen is van paternale oorsprong en dus 'vreemd') ook spelen cellen van het immuunsysteem een rol bij de rijping en groei van de placenta en foetus.

In deel II van de introductie wordt daarom de rol van het immuun systeem van de vrouwelijke voortplantings organen (ovaria, uterus) be-

schreven. De natural killer (NK) cellen blijken een belangrijke rol te spelen tijdens de implantatie. Ten eerste vormen de NK cellen de meerderheid van de witte bloedcel (leukocyten) populatie in het endometrium. Het percentage NK cellen neemt tijdens de menstruele cyclus snel toe en bereikt een piek in de luteale fase rondom de periode van innesteling. Tijdens het eerste trimester van de zwangerschap stijgt het aantal NK cellen verder tot wel 70% van alle leukocyten die aanwezig zijn in de decidua. Een tweede opmerkelijk feit is dat NK cellen accumuleren rondom de plaats van innesteling, zij worden dicht in de nabijheid van invasieve trophoblast cellen waargenomen. Een belangrijk aanknopingspunt voor verder onderzoek is dat NK cellen receptoren bevatten voor HLA-moleculen (HLA-G, HLA-E, HLA-CW) die aanwezig zijn op invasieve trophoblast cellen van de foetus. Er is steeds meer wetenschappelijk bewijs dat de interactie tussen de maternale NK cellen en invasieve trophoblast cellen van de foetus een cruciale stap is in het realiseren van een goede innesteling. Extravilleuze trophoblast cellen vervangen de spierwand van de spiraalarteriën, waardoor verwijding en ontwinding van deze arteriën optreedt. Daardoor kan een betere maternale bloeddoorstroming plaatsvinden naar de intervilleuze ruimte van de placenta. De meest gangbare hypothese is dat de (diepte van) remodellering van de spiraalarteriën door extravilleuze trophoblast cellen mogelijk wordt gemaakt door geactiveerde NK cellen.

Deel I

Single embryo transfer lijkt de beste strategie om het grote aantal meerlingen ten gevolge van IVF behandelingen drastisch te verminderen. Er zullen echter meer behandelingen met SET nodig zijn om, in vergelijking met DET, dezelfde zwangerschapskans te behalen. Aan de andere kant zullen er ook kosten bespaard worden door afname van maternale en perinatale complicaties veroorzaakt door meerlingzwangerschappen. Wij waren geïnteresseerd in de vraag hoeveel extra SET cycli nodig zijn om een evengrote zwangerschapskans te behalen als bij 1 DET cyclus en hoe groot de uiteindelijke kostenbesparing zou zijn na invoering van SET. Om die vragen te kunnen beantwoorden is in **hoofdstuk 2** berekend hoe duur een IVF tweeling zwangerschap is ten opzichte van een IVF éénling zwangerschap bezien vanuit medisch perspectief. De

belangrijkste 'cost drivers' tijdens de zwangerschap tot en met zes weken na de bevalling bleken de prenatale controles (1e of 2e lijns), de wijze van bevallen, ziekenhuis opname tijdens de zwangerschap en ziekenhuis opname van het kind(eren) (NICU en non-NICU opnames). De frequentie van voorkomen van deze 'cost drivers' werd bepaald bij 172 tweeling en 168 éénling zwangerschappen na IVF in het UMC St. Radboud. De kosten van deze 'cost drivers' werden berekend op basis van werkelijke kosten, indien mogelijk, of ziekenhuis tarieven. De medische kosten van een IVF tweeling zwangerschap bleken meer dan 5 keer zo hoog als die van een IVF éénling zwangerschap, respectievelijk 13.469 euro en 2.550 euro.

Vervolgens wordt in **hoofdstuk 3** de effectiviteit van single embryo transfer berekend. In een gerandomiseerde studie werd het aantal levend geborenen na 2 SET cycli vergeleken met het aantal levend geborenen na 1 DET cyclus. In totaal werden 107 IVF patiënten jonger dan 35 jaar, eerste behandeling en met tenminste één kwalitatief goed embryo, na stratificatie voor primaire of secundaire infertiliteit, gerandomiseerd. Het percentage levend geborenen na 2 opeenvolgende SET cycli was 41%. Dit percentage was vergelijkbaar met het aantal levend geborenen na 1 DET cyclus (36%), terwijl het aantal meerlingen in deze laatste groep significant hoger was: 37% in de DET groep en 0% in de SET groep ($p = 0.002$). Per levend geborene werden 1.5 x meer SET cycli dan DET cycli uitgevoerd. Wanneer de medische kosten van de extra IVF behandelingen en de kosten van IVF éénling en tweeling zwangerschappen tot en met zes weken postpartum bij elkaar werden opgeteld dan kostte de SET strategie 13.438 euro en de DET strategie 13.680 euro per levend geborene. Echter, wanneer de levenslange kosten van ernstig gehandicapte kinderen wordt meeberekend dan zal meer dan 7.000 euro bespaard worden door invoering van de SET strategie.

In **hoofdstuk 8** wordt bediscussieerd wat het theoretische effect zou zijn van de implementatie van SET bij patiënten met een gunstige zwangerschapsprognose op de gehele IVF populatie van het UMC St. Radboud in het jaar 2000 en 2001. Na extrapolatie van de gegevens van de SET-studie zou de overall zwangerschapskans slechts minimaal dalen van 23% naar 19%, terwijl de kans op een tweeling sterk zou afnemen van 27% naar 5%. Op landelijk niveau zou bijna 13 miljoen euro in 2 jaar tijd bespaard kunnen worden na invoering van SET, met een even hoge zwangerschapskans als bij DET.

Indien in de toekomst op steeds grotere schaal 1 embryo zal worden teruggeplaatst, dan moet ook serieus worden nagedacht over mildere ovariële hyperstimulatie schema's of zelfs géén hormonale stimulatie. Tot nu toe zijn er alleen studies verricht naar de effectiviteit van IVF in de natuurlijke cyclus, maar nog niet naar ICSI in de natuurlijke cyclus bij een serie paren waarvan de man extreem slechte zaadkwaliteit heeft. Terwijl juist in deze laatste groep er bij de vrouw geen sprake is van een aanwijsbare fertiliteitsstoornis en daardoor een hoge implantatiekans verwacht wordt. IVF/ICSI in de natuurlijke cyclus is een patiënt vriendelijke, relatief veilige en goedkope behandelingsstrategie. Bovendien kan deze vorm van behandeling op maandelijkse basis plaatsvinden, dit in tegenstelling tot een conventionele IVF behandeling welke 3 maanden in beslag neemt, omdat de eigen cyclus onderdrukt moet worden en de ovaria na stimulatie weer 'tot rust' moeten komen. Het nadeel van IVF/ICSI in de natuurlijke cyclus is de lagere zwangerschapskans per gestarte cyclus door een grote kans op canceling van de cyclus vanwege het ontbreken van een eicel of embryo. In **hoofdstuk 4** worden de resultaten van een pilotstudie naar de effectiviteit van ICSI in de natuurlijke cyclus bij 25 patiënten in 29 cycli beschreven. De inclusiecriteria waren extreem slechte zaadkwaliteit, leeftijd van de vrouw jonger dan 37 jaar, regulaire menstruele cyclus en geen aanwijsbare vruchtbaarheidsstoornis bij de vrouw. Het percentage verkregen eicellen na punctie was 58.6%. Alle met een zaadcel geïnjecteerde eicellen werden bevrucht. Bij 41.4% van de patiënten werd een embryo teruggeplaatst. Drie patiënten werden zwanger en bevielen van een gezonde éénling; dit is 10.3% per gestarte cyclus en 25% per embryo transfer. ICSI in de natuurlijke cyclus is een goed alternatief voor ICSI in de gestimuleerde cyclus, indien de cumulatieve zwangerschapskans van 3 natuurlijke cycli wordt vergeleken met 1 gestimuleerde cyclus.

Deel II

Het is bekend dat uteriene NK cellen van groot belang zijn voor goede innesteling van een embryo. Echter, NK cellen spelen mogelijk ook al een belangrijke rol in eerdere stadia van de voortplanting, vóór de implantatie. Wellicht oefenen leukocyten die aanwezig zijn in follikel vloeistof reeds invloed uit op de rijping en ontwikkeling van de oöcyten en de

embryo's die daaruit ontstaan. In **hoofdstuk 5** is onderzocht of de leukocyten samenstelling, met de focus op NK cellen, van follikel vloeistof van patiënten met een onbekende oorzaak van hun fertiliteitsstoornis anders is dan een controle groep bestaande uit patiënten met slechte zaadkwaliteit of tuba pathologie. Met behulp van flow cytometrie werden de aantallen T cellen, NKT cellen, NK cellen en subsets van NK cellen bepaald. Het belangrijkste resultaat van deze studie was dat in de groep met een idiopathische fertiliteitsstoornis er significant meer NK^{dim} ($CD56^{+}CD16^{+}$) cellen werden gevonden dan in de controle groep. NK^{dim} cellen hebben overwegend cytotoxische eigenschappen en zijn negatief geassocieerd met verscheidene reproductieve stoornissen, zoals habituele abortus. Deze overmaat van NK^{dim} cellen in follikel vloeistof zou oöcyt dysfunctie kunnen veroorzaken en dus mogelijk een verklaring kunnen zijn voor de 'onbekende' oorzaak van de fertiliteitsstoornis in deze groep patiënten.

In **hoofdstuk 6** is onderzocht of de leukocyten samenstelling van endometrium tijdens de implantatie fase verandert onder invloed van hormonale stimulatie van de ovaria. Ovariële hyperstimulatie veroorzaakt namelijk suprafysiologische concentraties van oestradiol en progesteron in het bloed. In de literatuur worden onderzoeken gerapporteerd die zowel gunstige als ongunstige effecten op de receptiviteit van het endometrium beschrijven. Zoals beschreven in **hoofdstuk 6** werd van 20 IVF patiënten (tijdens hormonale stimulatie) een endometriumbiopt (pipelle) genomen en werd de leukocyten samenstelling hiervan vergeleken met 18 biopten van vrouwen in de natuurlijke cyclus. Histologische datering wees uit dat de endometriumbiopten na IVF niet vaker uit fase waren dan de biopten in de controle groep. Flow cytometrische analyse van de leukocyten subsets liet een verhoogde ratio van uteriene NK^{bright} / NK^{dim} cellen zien, veroorzaakt door een relatief verminderd aantal NK^{dim} cellen ten opzichte van de controle groep. Een tweede opvallende bevinding van dit onderzoek was dat er een derde NK cel subset werd ontdekt; de $NK^{superbright}$ ($CD56^{+++}CD16^{-}$) cellen. Deze celpopulatie werd exclusief in het endometrium en niet in perifeer bloed gevonden. De specifieke functie van deze $NK^{superbright}$ subset moet nog verder onderzocht worden. Aangezien NK^{bright} cellen van het endometrium een positieve invloed hebben op de implantatie en NK^{dim} cellen negatief geassocieerd worden met reproductieve processen, wordt verondersteld dat hormonale stimulatie een gunstig effect kan hebben op de receptiviteit van het endometrium.

In **hoofdstuk 7** is onderzocht wat het effect is van de interactie tussen uterine NK cellen en HLA moleculen die aanwezig zijn op invaderende trophoblast cellen, nl. HLA-G en HLA-E. Een potentiële rol van uteriene NK cellen is de modulatie van implantatie door interactie met deze HLA-moleculen. In het onderzoek beschreven in **hoofdstuk 7** werden leukocyten geïsoleerd uit endometrium biopten van 13 fertiele vrouwen tijdens de implantatie fase. Deze leukocyten werden geïncubeerd met cellijnen getransfecteerd met HLA-G. Het bleek dat HLA-G de proliferatie en cytokine productie van uteriene NK cellen stimuleert. Terwijl er juist remming optrad van de proliferatie en productie van IFN-γ en IL-3 van de gehéle leukocyten populatie, voornamelijk bestaande uit T cellen. VEGF werd zowel door uteriene NK cellen als door de gehele leukocyten populatie uit het endometrium geproduceerd na interactie met HLA-G. Uit deze bevindingen zou voorzichtig geconcludeerd kunnen worden dat HLA-G een te sterke gegeneraliseerde respons van het maternale immuun systeem op de innesteling van de semi-allogene foetus onderdrukt, maar dat de specifieke functie van de uteriene NK cellen wel geactiveerd moet worden om goede invasie van trophoblast cellen mogelijk te maken. Het wordt steeds duidelijker dat met name HLA-G en HLA-E moleculen NK cellen aanzetten tot de productie van specifieke cytokines en angiogene factoren, waardoor adequate invasie van trophoblast cellen in de decidua kan plaatsvinden met als gevolg een goede aanleg van de placenta.

De implantatie van de foetus blijft voor een groot deel een immunologisch mysterie. In dit proefschrift is een klein facet, namelijk de rol van NK cellen tijdens de (pre)implantatie fase, onderzocht. Toekomstig onderzoek moet uitwijzen of de aan- of afwezigheid van NK cel subsets als prognostische marker voor de kans op zwangerschap kan fungeren. Manipulatie van de leukocyten samenstelling of de functie in follikel vloeistof en endometrium zou de kans op zwangerschap na IVF kunnen verbeteren. Een hoge zwangerschapskans is een belangrijke voorwaarde voor goede acceptatie van implementatie van single embryo transfer van IVF patiënten en IVF specialisten. SET is een kosten-effectieve methode om het grote aantal meerlingzwangerschappen na IVF drastisch te verminderen. Op landelijk niveau kan bijna 13 miljoen euro bespaard worden in 2 jaar tijd na invoering van SET, zonder dat dit ten koste gaat van de zwangerschapskans. Wij pleiten voor een vergoeding van de eerste

drie complete IVF behandelingen, inclusief terugplaatsing van cryo embryo's, op voorwaarde dat ten minste de eerste een SET behandeling zal zijn bij patiënten met een gunstige zwangerschapsprognose. Wanneer de eerste IVF behandeling niet vergoed wordt, zoals momenteel in Nederland het geval is, bestaat het gevaar dat zowel IVF patiënten als IVF specialisten sneller geneigd zijn om twee embryo's terug te plaatsen met alle medische, sociale en economische consequenties van dien.

Bibliography

Cloosterman SG, Hofland ID, **Lukassen HGM**, Wieringa MH, Folgering HTh, van der Heide S, Brunekreef B, van Schayck CP. House dust mite avoidance measures improve peak flow and symptoms in patients with allergy but without asthma: a possible delay in the manifestation of clinical asthma? J.Allergy Clin.Immunol. 1997;100(3):313-9.

van Lierop MJC, Wijnands F, Loke YW, Emmer PM, **Lukassen HGM**, Braat DDM, van der Meer A, Mosselman S, Joosten I. Detection of HLA-G by a specific sandwich ELISA using monoclonal antibodies G233 and 56B. Mol.Hum.Reprod. 2002;8(8):776-84.

Lukassen HGM, Kremer JAM, Lindeman EJM, Braat DDM, Wetzels AMM. A pilot study of the efficacy of intracytoplasmic sperm injection in a natural cycle. Fertility and Sterility 2003;79(1):231-2.

Lukassen HGM, van der Meer A, van Lierop MJC, Lindeman EJM, Joosten I, Braat DDM. The proportion of follicular fluid $CD16^{+}CD56^{DIM}$ NK cells is increased in IVF patients with idiopathic infertility. J.Reprod.Immunol. 2003;60(1):71-84

van der Meer A, **Lukassen HGM**, van Lierop MJC, Wijnands F, Mosselman S, Braat DDM, Joosten I. Membrane-bound HLA-G activates proliferation and interferon-gamma production by uterine natural killer cells. Mol.Hum.Reprod. 2004;10(3):189-95.

Lukassen HGM, Schönbeck Y, Adang EM, Braat DDM, Zielhuis GA, Kremer JAM. Cost analysis of singleton versus twin pregnancies after in vitro fertilization. Fertility and Sterility 2004;81:1240-6.

Lukassen HGM, Joosten I, Van Cranenbroek B, van Lierop MJC, Bulten J, Braat DDM, van der Meer A. Hormonal stimulation for IVF treatment positively affects the CD56bright/CD56dim NK cell ratio of the endometrium during the window of implantation. Mol.Hum.Reprod. 2004;10(7):513-20.

Lukassen HGM, Braat DDM, Wetzels AMM, Zielhuis GA, Adang EM, Scheenjes E, Kremer JAM. Two cycles with single embryo transfer versus one cycle with double embryo transfer: a randomized controlled trial. Hum.Reprod. 2005;20(3):702-8.

Dankwoord

Het schrijven van een proefschrift is nooit de verdienste van één persoon. Zonder de inzet, motivatie, kennis en steun van velen was het mij niet gelukt om tot dit eindresultaat te komen. Ik wil daarom iedereen die op welke manier dan ook heeft bijgedragen aan de totstandkoming van dit proefschrift heel hartelijk danken.

Allereerst gaat mijn dank uit naar mijn promotor Prof. dr. D.D.M. Braat. Beste Didi, jij zocht een onderzoeker die niet snel zou afhaken op een taai immunologisch onderwerp, mede dankzij jouw enthousiaste en motiverende manier van begeleiden ben ik zover gekomen. Bedankt voor je steun en vertrouwen in mij.

Mijn co-promotor Dr. I. Joosten wil ik danken voor de begeleiding van met name de immunologische onderzoeken. Beste Irma, ik heb veel geleerd van je deskundigheid op wetenschappelijk gebied. Je sociale interesse en gevoel voor humor zorgden voor een prettige werksfeer, waardoor ik mij direct thuis voelde op jouw afdeling. Hopelijk levert de samenwerking tussen de afdeling Bloedtransfusie en Transplantatie Immunologie (ABTI) en de afdeling Gynaecologie in de toekomst nog meer vruchtbaar onderzoek op.

Mijn co-promotor Dr. J.A.M. Kremer dank ik voor de begeleiding van de klinische onderzoeken. Beste Jan, jij had veel aandacht voor het belang van de toepasbaarheid van klinisch onderzoek in de praktijk. Deze brede klinische kijk op onderzoek was erg verhelderend en leerzaam voor mij.

Ik wil graag mijn mede-auteurs bedanken voor de goede samenwerking en hun bijdragen aan de verschillende artikelen: Alex Wetzels en Emiel Lindeman (IVF-lab), Eddy Adang (afdeling Medical Technology Assessment), Gerhard Zielhuis en Yvonne Schönbeck (afdeling Epidemiologie en Biostatistiek), Eduard Scheenjes (Ziekenhuis Gelderse Vallei, Ede), Hans Bulten (afdeling Pathologie), Arnold van der Meer en Bram van Cranenbroek (ABTI). De collega's van de research afdeling Farmacologie van Organon, Sietse Mosselman, Marie-José van Lierop en Frank Wijnands, wil ik in het bijzonder bedanken voor de waardevolle en prettige samenwerking.

Het promo-team van de ABTI (oud en nieuw): Hans, Jeroen, Peter en

Ellen. Bedankt voor de gezelligheid en de gastvrijheid. Het was altijd mogelijk om op jullie kamer onder het genot van een senseootje te werken. Bedankt ook voor het geduld om al het geklets tussen Arnold en mij aan te horen. Het kweeklab van de ABTI: Bram, Esther F., Jos, Esther v R., Ronald en Chris niet te vergeten. Ik heb jullie interesse voor mijn onderzoek en hulp in het kweeklab zeer gewaardeerd. Bram wil ik met name bedanken voor de praktische ondersteuning van de vele experimenten en kritische inbreng tijdens discussies. Boeiend vak hè, Gynaecologie!

Het IVF team wil ik hartelijk danken voor de ondersteuning en het meedenken over de uitvoering van de klinische onderzoeken. Als IVF-arts heb ik altijd met veel plezier in dit hechte team gewerkt.

Yvonne Schönbeck, stagiaire Biomedische Wetenschappen, wil ik danken voor de geweldige bijdrage aan hoofdstuk 2 van dit proefschrift. Veel succes met jouw promotie onderzoek.

Op mijn werkplek in de 'vissenkom' heb ik met Eva Maria, Jesper en René veel (onderzoeks) lief en leed gedeeld. Bedankt, jullie waren (en zijn) fijne collega's! Maar ik wil zeker ook de andere onderzoekers: Annemarie, Cathelijne, Erik, Iris, Marcel, Michael, Pascal, Petra, Ron, Ruben, Sabina, Tanya, Wai Yee en Willianne danken voor de leuke tijd. En natuurlijk Mark Flink, redder in computernood, fijn dat je me zo vaak geholpen hebt met die aanstekelijke big smile van je.

Tevens wil ik alle assistenten, gynaecologen, verpleegkundigen, polimedewerkers en secretaresses van de gynaecologie afdelingen van zowel het Radboud Ziekenhuis als het Rijnstate Ziekenhuis hartelijk danken voor hun interesse, steun en collegialiteit.

Arnold, jou wil ik speciaal bedanken voor het wegwijs maken in de wondere wereld van de immunologie. Je humor, steun en vriendschap waren erg belangrijk voor mij.

Ik wil mijn familie en vrienden bedanken voor hun voortdurende belangstelling in mijn onderzoek. Jullie zorgden voor veel afleiding en ontspanning, ook al moest ik de laatste tijd vaak verstek laten gaan. Fijn dat jullie zo begripvol waren.

Mijn ouders wil ik danken voor hun onvoorwaardelijke steun en liefde. Jullie hebben het mogelijk gemaakt en gestimuleerd om mijzelf maximaal te ontplooien. Lieve mam, erg fijn dat je tijdens mijn bevallingsverlof de zorg voor Eva met mij hebt gedeeld, zodat ik het proefschrift kon afronden.

Lieve Koen, als laatste wil ik jou bedanken. Jij hebt altijd achter mij gestaan en mij nooit beperkt in mijn werk. Dat heeft veel voor mij betekend. Jouw betrokkenheid en relativerend vermogen hebben er voor gezorgd dat het me is gelukt om te promoveren naast mijn opleiding. Nu gaan we samen volop genieten van onze dochter Eva!

Curriculum vitea

Marieke Lukassen werd geboren op 13 november 1970 te Arnhem. In 1989 behaalde zij haar VWO diploma aan het Liemers College te Zevenaar. Aansluitend startte zij met de studie Biomedische Gezondheidswetenschappen, afstudeerrichting epidemiologie, aan de Katholieke Universiteit Nijmegen (1989-1993). Deze studie resulteerde in de registratie als epidemioloog A. Na het behalen van haar doctoraalexamen besloot zij verder te studeren. In september 1993 startte zij met de studie Geneeskunde aan dezelfde faculteit (1993-1997). Tijdens deze studie volgde zij een studenten exchange project in een ziekenhuis in Lund, Zweden, op de afdeling Gynaecologie en Obstetrie. Daar werd haar interesse voor het specialisme Gynaecologie gewekt. Zij werkte tevens drie maanden als co-assistent op een gezondheidspost in Managua, Nicaragua.

Na het behalen van het arts examen werkte zij van maart 1998 tot april 1999 als assistent geneeskunde niet in opleiding (agnio) op de afdeling Gynaecologie en Obstetrie in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch. Vervolgens wilde zij weer wetenschappelijk onderzoek gaan verrichten. Vanaf april 1999 werd zij aangesteld als onderzoeker en IVF-arts op de afdeling Gynaecologie en Obstetrie van het Universitair Medisch Centrum St. Radboud te Nijmegen. In deze periode verrichtte zij onderzoek op de IVF-afdeling onder leiding van Prof. dr. D.D.M. Braat en Dr. J.A.M. Kremer en op de Afdeling Bloedtransfusie en Transplantatie Immunologie onder leiding van Dr. I. Joosten (ABTI, Prof. dr. B.E. de Pauw) wat resulteerde in dit proefschrift.

In juli 2002 begon zij met de opleiding Gynaecologie in het Universitair Medisch Centrum St. Radboud en het Rijnstate Ziekenhuis te Arnhem.

In augustus 2002 is zij getrouwd met Koen Rijnsaardt. Op 17 januari 2005 is hun dochter Eva geboren.